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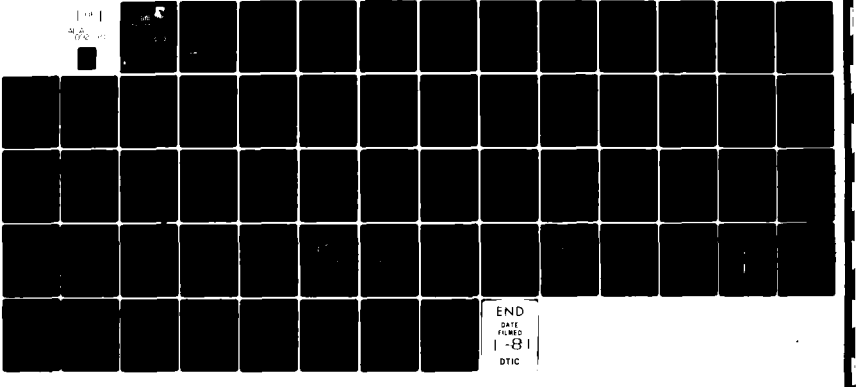

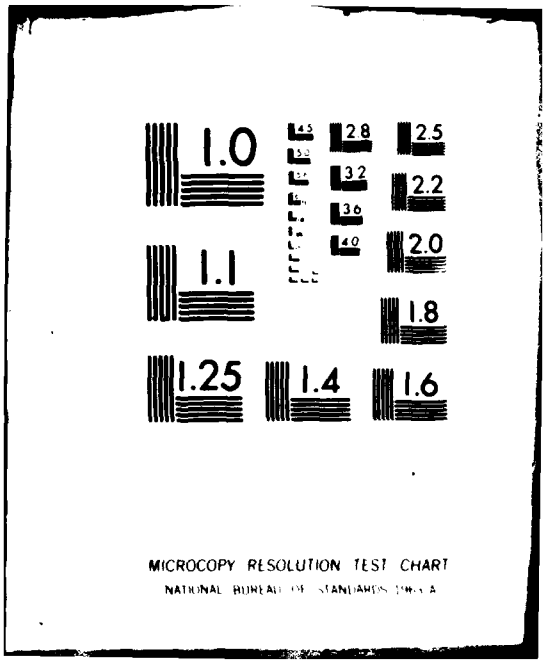
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COMPARATIVE METABOLISM OF HYDRAZINE AND NAPHTHALENE

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SEPTEMBER 1980

AIR FORCE AEROSPACE MEDICAL RESEARCH LABORATORY
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TECHNICAL REVIEW AND APPROVAL

AFAMRL-TR-80-103

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals, "Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

ANTHONY A. THOMAS, MD, SES
Director
Toxic Hazards Division
Air Force Aerospace Medical Research Laboratory

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PART I. PROPELLANT HYDRAZINES AND DNA METHYLATION

Hydrazine administration to rats and mice results in a rapid methionine-mediated methylation of nucleophilic sites in liver DNA similar to sites alkylated by chemical carcinogens; the aberrant DNA alkylation sites in hydrazine-treated animals have been identified as the 7- and O^6-positions of guanine. The hydrazine-provoked methylation is only weakly dose-dependent and active for less than 20 hours following a single administration of toxicant.

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The methylating agent in poisoned animals appears to be S-adenosylmethionine and not a methylation product of hydrazine itself (namely monomethylhydrazine). Ethionine, an antimetabolite of methionine, when given shortly before hydrazine, inhibits DNA methylation; however, if the ethionine precedes hydrazine by several hours, DNA methylation is enhanced. ~~Also~~, treatment of rats with small doses of ethionine soon after a large dose of hydrazine results in ethylation of DNA, an observation not expected for the amount of ethionine administered. Administration of other hepatotoxins, such as carbon tetrachloride or thioacetamide, also results in methionine-mediated methylation of liver DNA in rats, suggesting that the aberrant DNA methylation may be a response to toxic insult to the liver rather than a response specific to the chemical agent causing the insult. An in vitro method for measuring DNA methylation resulting from toxicant exposure has been developed.

PART II. NAPHTHALENE TOXICITY AND METABOLISM

Administration of doses of as little as 100 mg/kg naphthalene, a volatile hydrocarbon in shale oil, results in severe bronchiolar epithelial cell necrosis in mice. Necrosis in liver or kidney was not observed. This toxicity is prevented by pretreatment with an inhibitor of cytochrome P-450 and is increased substantially by pretreatment with a depletor of reduced glutathione. Administration of toxic doses of ^{14}C labelled naphthalene results in the covalent binding of radioactivity to tissue macromolecules which is greatest in tissues with high cytochrome P-450 activity and which is tripled by prior pretreatment with a glutathione depletor. This covalent binding is time dependent and precedes the development of tissue lesions. Pulmonary and hepatic but not renal glutathione is rapidly depleted by toxic doses of naphthalene. Continuing studies are examining the relationship between the pulmonary toxicity and the formation and fate of various naphthalene metabolites.

PREFACE

This is the annual report of the subprogram on Comparative Biochemistry and Metabolism and concerns work performed by the Department of Community and Environmental Medicine of the University of California, Irvine on behalf of the Air Force under Contract Number F33615-76-C 5005, Work Unit 2312V117. This document describes the accomplishments of the subprogram from June 1979 through May 1980.

R.C. Shank, Ph.D. and A.R. Buckpitt, Ph.D., served as co-coordinators for the subprogram. Acknowledgement is made to M.J. Oldham, R.A. Becker, W.S. Bosan and D.L. Brown, Jr., for their significant research contributions and assistance in the preparation of this report. K.C. Back, Ph.D., Chief of the Toxicology Branch, was the technical monitor for the Aerospace Medical Research Laboratory.

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PART I. PROPELLANT HYDRAZINES AND DNA METHYLATION

INTRODUCTION

Comparative metabolic studies (Shank, 1979) on the propellant hydrazines, hydrazine, monomethylhydrazine (MMH), and 1,1-dimethylhydrazine (UDMH) have concentrated on biochemical pathways considered relevant to chemical carcinogenesis. Most, if not all, strong carcinogens appear to act by altering the genetic structure of the target cell; the process involves the spontaneous or metabolic transformation of the carcinogenic chemical to a reactive electrophile which binds covalently to nucleophilic sites in DNA. The propellant hydrazines have been reported to be carcinogenic, and thus, the research focus in the studies of this subprogram has been on formation of DNA adducts following the administration of the hydrazines.

In the course of these studies hydrazine administration to rats and mice resulted in the methylation of DNA at sites usually attacked by strong carcinogens; the methylation appeared to utilize S-adenosylmethionine and manifest a cellular response to hydrazine rather than a direct action of a hydrazine metabolite (Shank, 1979). This was the first evidence to relate hydrazine toxicity to a biochemical process potentially relevant to carcinogenesis, and DNA methylation resulting from the administration of hydrazine, MMH, and UDMH has been the emphasis of the continuing studies.

RESEARCH PROGRAM

METHODS DEVELOPMENT

Determination of the Specific Activity of S-Adenosylmethionine in Liver

The studies on the methylation of liver DNA in animals treated with hydrazine and radiolabeled methionine required the determination of the specific activity of S-adenosylmethionine.

Rats were decapitated at various intervals after receiving intraperitoneally 100 μCi ^{3}H-methyl-methionine in 0.1 ml saline. Livers were homogenized individually in one volume of ice cold water and S-adenosylmethionine was extracted from the homogenate with a volume of 68% perchloric acid equal to two times the original tissue weight; extraction was carried out in 50-ml Teflon centrifuge tubes, under nitrogen for 10 minutes on a wrist-action shaker. The mixture was centrifuged at 10,000 g for 20 minutes and the volume of supernatant was measured. To the isolated supernatant, 1/4

volume of saturated K_2HPO_4 was added to precipitate the perchlorate; this procedure was done in the cold. The mixture was centrifuged at 10,000 g for 10 minutes and the supernatant was filtered (0.65 μm pore size) prior to injection into the liquid chromatograph (HPLC). (The S-adenosylmethionine extracts were stable for 5 days following perchlorate removal.)

Two successive injections of 1 ml each were made onto the HPLC under dilute acetic acid (pH 3) using a Whatman preparative strong cation exchange 50 cm column; elution with dilute acetic acid (4 ml/min) continued for 20 minutes before the mobile phase was changed to 0.1 M ammonium phosphate, pH 3. Elution of fractions was monitored by ultraviolet absorption at 260 nm. S-Adenosylmethionine eluted as a shoulder on the tail of a large fraction (Figure 1) and was collected in the elution volume (176-204 ml) 44-52 minutes after injection. This partially purified S-adenosylmethionine fraction was reduced to a 1 ml volume in a rotary evaporator at 40°C and reinjected onto the preparative cation exchange column under dilute acetic acid (pH 3). The mobile phase was immediately changed to 0.1 M ammonium phosphate pH 3 to elute the S-adenosylmethionine (Figure 2). The amount of S-adenosylmethionine was determined graphically measuring the area under the chromatographic peak and comparison to elution of known amounts of S-adenosylmethionine. The amount of radioactivity in the elution volume was determined by standard liquid scintillation techniques, and the specific activity was calculated as the dpm (disintegrations/minute) per μg S-adenosylmethionine. Liver homogenates were spiked with ^{14}C-methyl-S-adenosylmethionine to determine that the recovery of the amino acid derivative by this method was 41.2%.

GC/MS Analysis of Purines and Pyrimidines

A method has been developed to confirm the identity of alkylated bases in DNA using gas chromatography and mass spectrometry. The method was described by Shank (1979) and gave GC/MS characteristics for derivatized thymine, cytosine, 5-methylcytosine, uracil, adenine, guanine, 7-methylguanine, O^6-methylguanine, O^6-ethylguanine, and N^6-methyladenine. The method is applicable to analysis of 7-ethylguanine.

The technique involves silylation of purified bases isolated from DNA extracted from animals treated with alkylating agents; individual bases are isolated from DNA hydrolysate by reverse phase high pressure liquid chromatography. On a preparative scale 300 μg of 7-ethylguanine (Vega-Fox, Tucson, Arizona) in 300 μl butanol:acetic acid:water (24:30:50, v:v:v) were transferred to a silylation vial and dried under warmed nitrogen; to the vials were added 150 μl acetonitrile and 150 μl BSTFA (bis-trimethylsilyl trifluoracetamide). The vials were sealed and heated in a sand bath at

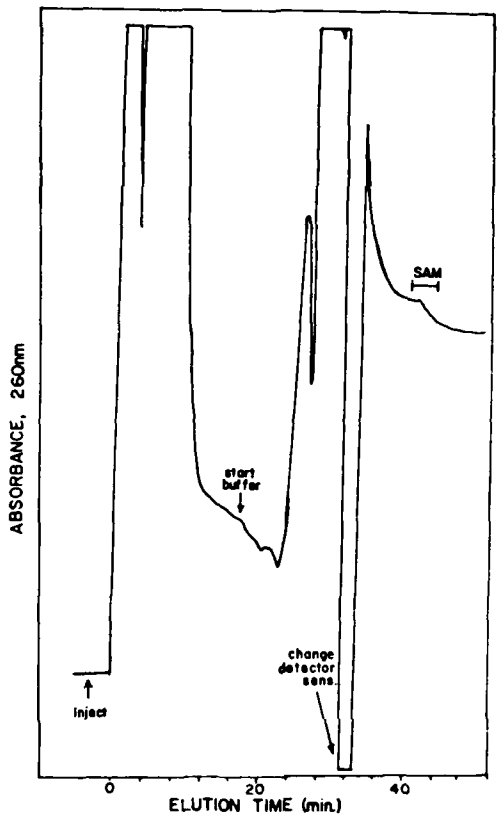

Figure 1. Preparative chromatographic separation of S-adenosylmethionine from perchloric acid extract of rat liver homogenate.

150°C for 30 minutes and then cooled to room temperature. The silylated mixture (2-5 µl) was injected onto a gas chromatographic column (3% OV-1, 80/100 WHP, 4 ft, Hewlett Packard) interfaced with a mass spectrometer (Hewlett Packard GC/MS Model 5992A). Instrument conditions were as follows: GC - helium flow of 30 ml/minute, injection port temperature of 225°C, initial column temperature of 90°C which was held constant for 4 minutes then increased to 280°C at a programmed rate of 8° per minute; MS - tuned automatically using perfluorotributylamine as the calibrating standard, atomic mass unit scanning range of 10 to 500 at a rate of 690 scans per second.

The base, 7-ethylguanine, took on two trimethylsilyl groups, but a molecular ion, 323 amu, was not obtained; the major fragments were:

Fragment	Mass	% Rel. Abundance
trimethylsilyl	73	100
7-ethylguanine (7EG)	179	27
monosilyl-7EG less CH_3	236	83
monosilyl-7EG	251	70
disilyl-7EG less CH_3	308	13

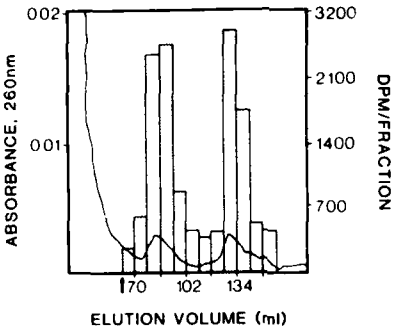

Figure 2. Elution of purified rat liver S-adenosylmethionine by high pressure liquid chromatography. Arrow indicates time at which fraction collection began for radioactivity determinations (stippled bars); curve represents elution of ultraviolet-absorbing material with S-adenosylmethionine eluting between 70 and 102 ml.

Disilyl-7-ethylguanine had an elution time of 22.4 minutes (247°C) under these chromatographic conditions. Table 1 summarizes the GC/MS characteristics of the eleven silylated pyrimidines and purines analyzed to date.

Limits of Detection in GC/MS Analysis of Methylated Purines

The limits of detection of three methylated purines and pyrimidines were determined for the gas chromatography-mass spectrometry identification method. Reference bases were silylated before GC/MS analysis; 100 μg of thymine, adenine, 7-methylguanine, and O^6-methylguanine were transferred to silylation vials to which were added 50 μl BSTFA and 50 μl acetonitrile. The vials were heated to 150°C for 30 minutes, and when cooled, the mixtures were serially diluted with acetonitrile. Each dilution was analyzed by GC/MS using specific ion monitoring: oven temperature 90°C

TABLE 1

GAS CHROMATOGRAPHIC AND MASS
SPECTRAL CHARACTERISTICS OF
SILYLATED PYRIMIDINES AND PURINES

Silylated Base	Elution Time, min	Base Peak	Mol. Wt.
Thymine (disilyl)	12.9	255	270
Cytosine (disilyl)	14.8	73	255
Cytosine (trisilyl)	16.6	73	327
5-Methylcytosine (disilyl)	15.3	254	269
Uracil (disilyl)	12.3	241	256
Adenine (disilyl)	19.5	264	279
Guanine (trisilyl)	22.6	73	367
7-Methylguanine (disilyl)	23.1	294	309
7-Ethylguanine (disilyl)	22.4	73	323
O^6-Methylguanine (monosilyl)	21.6	222	237
O^6-Methylguanine (disilyl)	22.4	73	309
O^6-Ethylguanine (monosilyl)	23.1	208	251
O^6-Ethylguanine (disilyl)	23.8	208	323
N^6-Methyladenine (monosilyl)	18.4	221	221
N^6-Methyladenine (disilyl)	20.3	278	293

for 4 minutes then programmed to increase 8°/minute to 280°C, carrier gas was He, 30 ml/minute, port temperature was 225°C, 3% OV-1, 80-100 mesh, 4 ft. column. The limits of detection were as follows:

silylated thymine	0.15 µg/20 µl injection
silylated adenine	0.20 µg/20 µl injection
silylated O^6-methylguanine	0.25 µg/20 µl injection

If one were to attempt GC/MS confirmation of the minor component, O^6-methylguanine, in liver DNA from rats treated with 60 mg hydrazine per kg body weight (which produces 20 µmoles O^6-methylguanine/mole DNA guanine 13 hours after hydrazine administration), the O^6-methylguanine from at least 150 mg of DNA (75 g liver) would be needed for analysis by this technique.

Determination of $^{15}N/^{14}N$ in Liver DNA

Experiments are planned to determine if ^{15}N is incorporated into DNA in animals given ^{15}N-hydrazine; if such incorporation is detected, efforts will be made to determine the chemical nature of the ^{15}N-DNA adduct(s).

Liver DNA was digested by the standard Kjeldahl technique to convert all DNA nitrogen to ammonium sulfate, which was subsequently distilled as ammonia and trapped in aqueous solution. Several attempts to react the ammonia with alkyl halides to form alkyl amines reproducibly and quantitatively were unsuccessful; poor reaction rates were due primarily to two-phase separation of reactants.

Direct analysis of DNA digests for ammonia appear to be more appropriate. Ammonia can be distilled from the digest and trapped in the cold (liquid N_2); the trap is then sealed and allowed to come to room temperature. The head space in a model system was sampled with a gas-tight syringe and analyzed by GC/MS. The column used was a 3 ft Porapak N, 80/100 mesh which proved unable to fully resolve ammonia and water. Work is continuing to improve chromatographic conditions for sensitive mass spectral analysis of the ammonia from DNA nitrogen.

HYDRAZINE METABOLISM

Shank (1979) reported previously that rats given 60 mg hydrazine per kg body weight [approximately a $LD_{0.01}$, estimated from dose lethality data analyzed according to Litchfield and Wilcoxon (1949)] rapidly methylate liver DNA, that the methylation sites involve not only the normal 5-position of cytosine but also the 7- and O^6-positions of guanine, and that the methylation process is mediated via methionine and presumably S-adenosylmethionine. This aberrant methylation has been the focus of several experiments.

Effect of Hydrazine Dose on DNA Methylation in the Rat

The first study was an attempt to determine whether DNA methylation in hydrazine-treated rats was related to the dose of toxicant administered, to demonstrate a response specific to hydrazine itself. Young adult male Fischer 344 rats were fasted overnight and given by intubation 30.0, 42.4, 60.0, or 84.9 mg hydrazine per kg body weight (LD_{50} is 80 mg/kg body weight) in 0.1 ml of 0.1 M HCl per 100 g body weight. Immediately following hydrazine administration, 100 µCi ^3H-methyl-methionine were injected intraperitoneally in 0.1 ml 0.9% NaCl, and these methionine injections were repeated hourly until the animals were killed 5 hours after hydrazine administration. Control animals were given 0.1 M HCl p.o. and radiolabeled methionine i.p. only.

Two animals given each dose were decapitated 5 hours after hydrazine administration, and liver and lung DNA were isolated by the phenolic extraction technique of Swann and Magee (1968). DNA samples were partially hydrolyzed in 0.1 M HCl at 70°C for 30 minutes to produce the pyrimidine oligonucleotides and free purine bases. The hydrolysates were fractionated by high pressure liquid chromatography to quantitate the amounts of methylated bases in DNA. Hydrolysates, containing added standard 7-methylguanine and O^6-methylguanine as carrier were filtered and fractionated on a Micromeritics Model 7000B liquid chromatograph using a Whatman Partisil M9 10/50 preparative strong cation exchange column. Pyrimidine oligonucleotides were eluted with water, and a mobile phase of 0.1 M ammonium phosphate pH 2.0 at 4 ml/min was used to separate the individual purine bases. Five ultraviolet-absorbing (determined at 275 nm) fractions, representing pyrimidine oligonucleotides, guanine, adenine, 7-methylguanine, and O^6-methylguanine, and the eluate between these fractions ("troughs") were collected. For analysis of 5-methylcytosine, pyrimidine oligonucleotide fractions were dried in a rotary evaporator, and hydrolyzed in 2 ml of 68% perchloric acid (1 ml/5 mg DNA) at 100°C for 1 hour. After hydrolysis, perchloric acid was precipitated by addition of saturated K_2HPO_4 (1 ml/2 ml acid) and removed by centrifugation, and the supernatant was adjusted to pH 7 with 50% KOH (ca. 0.25 ml/ml acid). Pyrimidine bases were filtered (0.65 μm pore size) and fractionated by high pressure liquid chromatography using the above instrument and column. Pyrimidines were eluted with 0.1 M ammonium phosphate pH 2.0 at 4 ml/minute and detected by their absorption at 275 nm. Each fraction was concentrated on a Dowex 50 8x H^+ cation exchange column, eluted with 0.1 M ammonium acetate, pH 10, and counted for radioactivity by standard liquid scintillation techniques. The amounts of methylated bases in DNA samples were estimated by assuming the specific activity of each methylated purine or pyrimidine was the same as that of the probable methyl donor, S-adenosylmethionine; the specific activity of S-adenosylmethionine was estimated according to the method of Craddock (1974).

Liver DNA from hydrazine-treated rats contained up to three methylated bases when nucleic acid hydrolysates were analyzed by high pressure liquid chromatography; lung DNA from hydrazine-treated rats and all control-rat DNA (liver and lung) contained only 5-methylcytosine (the only normal methylated base in mammalian DNA).* The three bases in liver DNA isolated from poisoned rats were 5-methylcytosine, 7-methylguanine and O^6-methylguanine (Figure 3). There appears to be only a weak dose-response relationship for hydrazine and aberrant methylation of DNA, suggesting that this response is nearly maximal at the lowest dose of hydrazine administered. The methylation of DNA at the 5-position of cytosine was altered (increased) only at the highest dose of hydrazine studied, approximately the 7-day LD_{50}.

* Efforts were not successful in isolating DNA from rat nasal turbinate, a tissue sensitive to the carcinogenicity of hydrazine in an inhalation study (MacEwen et al., 1980).

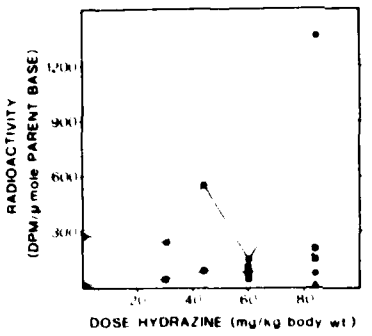

Figure 3. Effect of hydrazine dose on liver DNA methylation in the rat. DNA from hydrazine-poisoned animals contained 5-methylcytosine (closed circles), 7-methylguanine (closed squares) and O^6-methylguanine (closed triangle).

Time-Response Relationship Between Hydrazine and DNA Methylation in the Rat

Several experiments were performed to determine how rapidly hydrazine toxicity produced aberrant methylation of DNA and at what time after hydrazine administration the methylation response was maximal; for these studies a dose of 60 mg hydrazine per kg body weight (estimated $LD_{0.01}$) was used.

Fasted rats were given hydrazine by intubation and hourly intraperitoneal injections of ^3H-methyl-methionine as described in the dose-response study. Two animals were decapitated at 0.5, 1, 3, 5, 9, and 13 hours after hydrazine administration; in an additional group of animals hourly methionine injections were delayed 19 hours and the animals were killed 24 hours after toxicant administration. Isolated liver DNA was analyzed for the presence of methylated bases by high pressure liquid chromatography.

The results are shown in Figure 4. The methylation of guanine occurred soon after hydrazine administration and increased slowly over several hours; the response appeared to be short-lived, as aberrant methylation of DNA 19 hours after hydrazine treatment could not be demonstrated although the methylation at the 5-position of cytosine was normal. Trace amounts of radioactivity cochromatographing with standard O^6-methylguanine were detected in liver DNA hydrolysates 9 and 13 hours after hydrazine administration. The level of O^6-methylguanine in DNA from hydrazine-treated rats may parallel the level of 7-methylguanine, reaching detection limits of the chromatographic system only after 9 hours post-treatment; alternatively, the methylation of DNA guanine in poisoned rats may proceed by separate mechanisms.

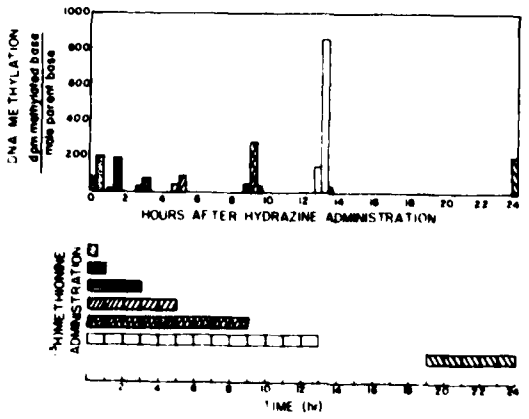

Figure 4. Variation with time in methylation of liver DNA in hydrazine-treated rats. Hourly intraperitoneal injections of ^{3}H-methyl-methionine were administered to rats *immediately after a single oral dose of hydrazine* except for one group of animals for which methionine injections did not begin until 19 hours after hydrazine administration. Each pair of bars in the top chart represents radioactivity in 7-methylguanine and 5-methylcytosine, respectively, in DNA from pooled livers from 3 rats; the single bar at 24 hours represents radioactivity in 5-methylcytosine only as no 7-methylguanine was detected. Detection of trace amounts of radioactivity associated with O^{6}-methylguanine is indicated by X at 9 and 13 hours. The bottom chart illustrates administration of hourly pulses of methionine which are associated graphically with particular DNA methylation patterns in the top chart by coordinated hatching of the bars.

Duration of DNA Methylation Process in Hydrazine-Treated Rats

To determine more accurately the period of time during which the active methylation of guanine in liver DNA takes place in hydrazine-treated rats, a series of experiments was begun in which the interval between hydrazine administration and labeling of the methionine pool was varied. In the first experiment two rats were given orally 60 mg hydrazine per kg body weight and two hours later 100 μCi (^{3}H)-methyl-methionine intraperitoneally. The methionine injections were repeated hourly until the animals were decapitated 9 hours after hydrazine administration. Liver DNA was isolated and assayed for the presence of 7-methylguanine and O^{6}-methylguanine using

cation exchange high pressure liquid chromatography. The DNA contained 87 dpm as 7-methylguanine per mole guanine and 9 dpm as O^6-methylguanine per mole guanine, demonstrating that the aberrant methylation of liver DNA in hydrazine-treated rats is still active 2 hours after toxicant administration; thus, the methylation process is not limited to a short burst of activity.

The experimental design was changed in the second study to provide for a more accurate determination of the period during which DNA methylation was active after hydrazine administration and to allow correlation during hydrazine-induced hepatotoxicity. The correlation is relevant because normal DNA methylation takes place shortly after DNA synthesis (Burdon and Adams, 1969; Craddock, 1970), and it is conceivable that the aberrant methylation of DNA in hydrazine-treated rats may be a manifestation of aberrant activity in the normal methylation process.

The new design required that a single pulse of ^{14}C-methyl-methionine be administered simultaneously with a single pulse of ^{3}H-methyl-thymidine to rats at various intervals after intubation with 60 mg hydrazine/kg body weight. DNA methylation at one interval after hydrazine administration is measured in each experiment, beginning with 1 hour after hydrazine and progressing hourly, until no further aberrant DNA methylation can be detected up to 20 hours after hydrazine, if necessary. The animals are decapitated 24 hours after hydrazine administration and liver DNA is isolated for methylation and thymidine incorporation analysis. The simultaneous use of methionine and thymidine permits comparison of the kinetics of the DNA methylation process with the kinetics of DNA synthesis (and repair, if any).

A preliminary experiment was done to determine appropriate amounts of radioactivity for the injections and whether fasting the animals had any effect on DNA methylation in hydrazine-treated rats. Since the animals are to be held 24 hours after hydrazine administration in the definitive experiments, it would be desirable not to fast them prior to treatment or maintain fasting throughout the experiment.

Four male Fischer 344 rats (Charles River) were divided into two groups. Two rats were fasted overnight while the second group was given chow ad libitum. On the morning of the experiment both groups were given 60 mg hydrazine/kg body weight in 0.1 M HCl by intubation. Immediately thereafter each animal in both groups was given first an intraperitoneal injection of 50 μCi ^{14}C-methyl-methionine (13.8 mCi/mmol), followed by an intraperitoneal injection of 500 μCi ^{3}H-methyl-thymidine (82.1 Ci/mmol); 30 minutes later a second injection of 500 μCi ^{3}H-methyl-thymidine was given, as the total injection volume for thymidine was 1 ml, too large for a single injection. The rats were decapitated 24 hours after hydrazine administration and the livers from the two animals in each group were pooled for DNA isolation. The DNA was hydrolyzed in 0.1 M HCl for

30 minutes at 70°C and fractionated by standard strong cation exchange high pressure liquid chromatography. The results are summarized in Table 2.

TABLE 2

INCORPORATION OF RADIOLABEL FROM ^{3}H-METHYL-THYMIDINE AND ^{14}C-METHYL-METHIONINE INTO LIVER DNA OF RATS TREATED WITH HYDRAZINE

DNA Base*	Radioactivity, dpm/mg DNA			
	Fasted Rats		Fed Rats	
	^{3}H	^{14}C	^{3}H	^{14}C
Thymine	1912	125	2845	85
Cytosine	29	69	44	21
5-Methylcytosine	8	44	11	38
MP2**	39	64	22	47
Guanine	5	24	8	28
Adenine	1	34	56	5
7-Methylguanine	1	35	2	16
Trough	4	5	3	4
O^{6}-Methylguanine	1	1	1	1

* in chromatographic order; pyrimidines and MP2 fractionated by a system different from that used to fractionate purines

** unidentified elution fraction

Methionine and thymidine have short half-lives in vivo and their respective metabolic pools in liver are labeled by a single pulse for not much longer than an hour. Finding 7-methylguanine in liver DNA of hydrazine-treated rats by the technique used in this experiment suggests an almost immediate DNA methylation following hydrazine administration. Fasting the animals reduced thymidine incorporation into liver DNA, presumably due to reduced protein synthesis; the reduced incorporation of radiolabel (^{14}C) into 5-methylcytosine (compare 5-methylcytosine with cytosine) in fasted rats is consistent with decreased DNA synthesis. The formation of 7-methylguanine, however, appears to be greater in fasted animals; this observation does not support a link between aberrant DNA methylation and DNA synthesis in the hydrazine-poisoned rat.

Confirmation of Identity and Quantitation of 7-Methylguanine and O^6-Methylguanine in Liver DNA from Hydrazine-Treated Rats

A large quantity of liver DNA from hydrazine-treated rats was prepared to allow mass spectrometric confirmation of the identity of 7-methylguanine isolated from the DNA. Twenty fasted young male Fischer 344 rats were given orally 60 mg hydrazine per kg body weight and decapitated 13 hours later. A small amount of the 104 mg DNA isolated from 119 gm pooled livers was hydrolyzed in 0.1 M HCl and analyzed for the presence of methylated bases using the highly sensitive fluorescence technique of Herron and Shank (1979): DNA (13.3 mg) was hydrolyzed in 2 ml 0.1 M HCl for 40 minutes at *70°C, with no carrier added; 1 ml of the filtered hydrolysate was applied to a* cation exchange preparative column and eluted with 0.045 M ammonium phosphate pH 2.3, 4 ml/minute, with fluorescence detection at 286 nm excitation, 366 nm interference emission filter. The chromatogram (Figure 5) clearly demonstrates the presence of 7-methylguanine and O^6-methylguanine using optical rather than radioisotope techniques. The DNA contained an estimated 300 µmoles 7-methylguanine and 20 µmoles O^6-methylguanine per mole guanine. This level for 7-methylguanine is approximately 2.5 times greater than that calculated by estimating the specific activity of the S-adenosylmethionine pool label after ^{3}H-methyl-methionine administration. This difference would indicate an overestimation of the S-adenosylmethionine specific activity following radiolabeled methionine administration to rats, thus underestimating the amount of methylated base formed.

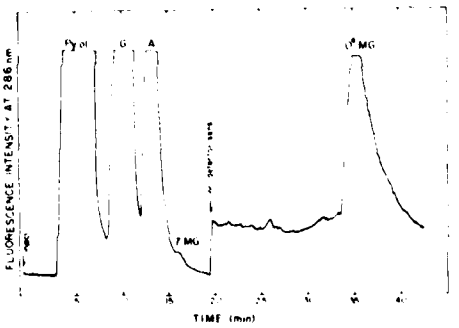

Figure 5. Elution profile of pyrimidine oligonucleotides and purines from liver DNA hydrolysate prepared from a hydrazine-treated rat. *Py. ol., pyrimidine oligonucleotides;* G, guanine; A, adenine; 7-MG, 7-methylguanine; O^6-MG, O^6-methylguanine; sensitivity of the fluorescence detector was increased approximately 20 minutes after injection of hydrolysate.

Specific Activity of the Hepatic S-Adenosylmethionine Pool in Rats Given a Single Pulse of ^{3}H-Methyl-Methionine

Craddock (1974) demonstrated that the turnover of S-adenosylmethionine in rat liver is rapid, and that the radiolabel from methionine in the hepatic S-adenosylmethionine pool is detectable for no longer than about one hour after administration of a single pulse of the amino acid. An experiment was carried out to determine whether the turnover of radiolabel from methionine was also rapid in S-adenosylmethionine pools in hydrazine-treated rats and whether the specific activity of this pool could be used to calculate the levels of methylated bases in liver DNA.

Rats were given 100 µCi ^{3}H-methyl-methionine in 0.1 ml saline intraperitoneally following oral administration of 60 mg hydrazine/kg body weight in 0.1 M HCl or only the HCl (control animals). Three control animals were decapitated 15, 35, and 60 minutes after injection of the methionine and hepatic S-adenosylmethionine was isolated for specific activity determination as described in Methods. The results, given in Table 3, suggested that the maximum specific activity of the S-adenosylmethionine may have occurred before the earliest measurement (15 minutes) after methionine administration; therefore, in the second part of the experiment, hydrazine-treated animals were killed 10, 20, 30, 45, and 60 minutes after methionine administration. The results (Table 3) indicated that not only was more radioactivity recovered in S-adenosylmethionine at the earliest measurement (10 minutes) but also less S-adenosylmethionine was recovered; animals treated with hydrazine had only 27% of the amount of S-adenosylmethionine 10 minutes after methionine administration as did control animals 15 minutes after receiving the amino acid. The S-adenosylmethionine levels in livers of hydrazine-treated rats returned to near control levels 20 minutes after hydrazine administration but appeared to remain slightly deficient throughout the one-hour study. The nature of the apparent transient depletion of hepatic S-adenosylmethionine in hydrazine-treated rats remains to be explored. The specific activity of S-adenosylmethionine in hydrazine-treated rats varied little from the levels measured in control rats, with the exception of the earliest measurements; those results are summarized in Figure 6.

Monomethylhydrazine As An Intermediate In DNA Methylation In Hydrazine-Treated Rats

One mechanism by which liver DNA could be methylated in hydrazine-treated rats is for the toxicant to be enzymatically methylated by S-adenosylmethionine to form MMH. Hawks and Magee (1974) have shown that MMH indirectly methylates rat liver DNA, and a few instances have been documented describing the in vivo methylation of primary amine compounds, such as norepinephrine, guanidoacetic acid and dimethylaminoethanol (Greenberg, 1963; Mandel, 1971).

TABLE 3

SPECIFIC ACTIVITY OF HEPATIC S-ADENOSYLMETHIONINE (SAM) AND HYDRAZINE-TREATED RATS FOLLOWING ADMINISTRATION OF A SINGLE PULSE OF ^{3}H-METHYL-METHIONINE

Treatment	Time After Methionine and Hydrazine (min)	dpm SAM Peak*	µg SAM peak*	dpm µg SAM
Control	15	2,930	2.7	3,177
	15	2,854	2.5	3,341
	15	1,583	3.2	1,583
	35	2,007	2.7	743
	35	1,407	3.9	361
	35	2,881	3.7	779
	60	90	2.8	32
	60	907	2.6	349
	60	761	2.3	331
Hydrazine	10	6,126	0.7	8,751
	10	14,892	0.8	18,615
	20	5,322	3.9	1,364
	20	4,470	1.9	2,352
	30	4,360	2.7	1,614
	30	2,380	1.7	1,400
	45	4,493	3.2	1,404
	45	1,218	1.5	812
	60	768	2.4	320
	60	890	1.6	556

* Chromatographic elution peak

Rats were given 15 mg MMH/kg body weight orally in 0.1 M HCl and decapitated 13 hours later; no methionine was given to these animals. The fluorescence liquid chromatographic technique of Herron and Shank (1979) was used for the optical detection of methylated bases in 19.6 mg liver DNA isolated from MMH-treated rats. No 7-

methylguanine or O^6-methylguanine was detected, whereas in a similar experiment analysis of 13.3 mg liver DNA from rats treated with 60 mg hydrazine/kg body weight produced readily detectable methylated guanines (Figure 5).

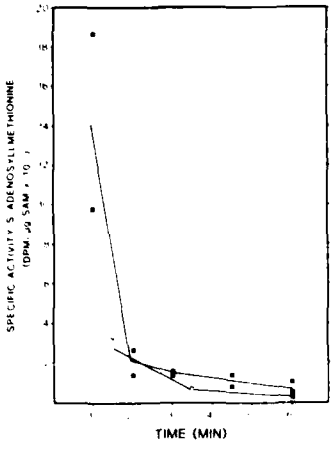

Figure 6. Effect of hydrazine on the specific activity of S-adenosylmethionine after administration of a single pulse of ^{3}H-methyl-methionine. Control rats (open circles); hydrazine-treated rats (closed squares).

The extent of DNA methylation of MMH was considerably less than the methylation seen with hydrazine; however, the doses of the two hydrazine compounds were equitoxic, not equimolar. The dose of MMH, equimolar to the hydrazine dose (1.88 mmoles/kg), was neurotoxic, rapidly producing convulsions and death, precluding the definitive study in the rat. Studies using equimolar doses of hydrazine and MMH were done in the mouse.

Liver DNA Methylation in the Hydrazine-Treated Mouse

The studies on DNA methylation following hydrazine administration were extended to the mouse to determine whether the methylation response was evident in more than one species and to facilitate comparative methylation studies on hydrazine and MMH.

Groups of 10 mice were intubated with 5.0, 7.1, 10.0, and 14.4 mg hydrazine per kg body weight in 0.1 M HCl, 0.04 ml per 20 g body weight; at the same time, 20 μCi ^{3}H-methyl-methionine in 0.03 ml of 0.9% NaCl were given intraperitoneally, and these injections were repeated hourly until the animals were killed 5 hours after hydrazine administration. Control animals were intubated with 0.1 M HCl and received ^{3}H-methyl-methionine intraperitoneally as described above.

Similarly, mice were given 10 mg hydrazine per kg body weight approx. $LD_{0.01}$ estimated from dose lethality data analyzed by the method of Litchfield and Wilcoxon (1949) and ^{3}H-methyl-methionine hourly as above. Animals were killed in groups of 10 at 0.5, 1, 3, 5, 9, and 13 hours after hydrazine administration.

Figure 7 summarizes the results of the dose-response study, showing the levels of methylated guanine and cytosine detected 5 hours after hydrazine administration. The results are similar to those obtained in the rat, little increase in the amount of 7-methylguanine produced with an almost 3-fold increase in dose; no O^6-methylguanine was detected. In the time-response study, Figure 8, the results closely paralleled those observed with the rat. The methylation of guanine occurred rapidly after administration of hydrazine and increased slowly over the next several hours. A trace amount of O^6-methylguanine was detected 13 hours after poisoning. Except for a possible increase in the amount of methylation of cytosine immediately after hydrazine administration, the levels of 5-methylcytosine in DNA from treated and control animals were in close agreement.

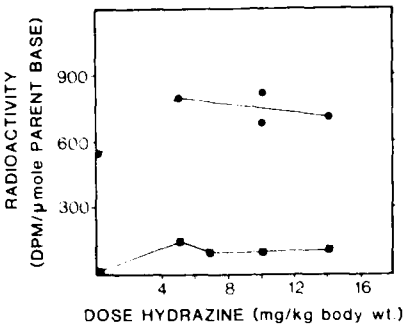

Figure 7. Effect of hydrazine dose on liver DNA methylation in the mouse. DNA from hydrazine-poisoned animals contained 5-methylcytosine (closed circles) and 7-methylguanine (closed squares).

Results on DNA methylation resulting from MMH administration to mice are presented later in this report.

Effect of Hydrocortisone on DNA Methylation Following Hydrazine Administration

In 1976 Kudryashova and Vanyushin demonstrated an approximate twofold increase in liver DNA cytosine methylation 40 minutes after hydrocortisone administration to rats; the increased methylation involved enzyme activation and an increase in the number of methylation sites available in liver DNA. It was suggested (Shank, 1979) that hydrazine toxicity might increase the level of circulating corticosteroids which in turn might alter DNA methylation.

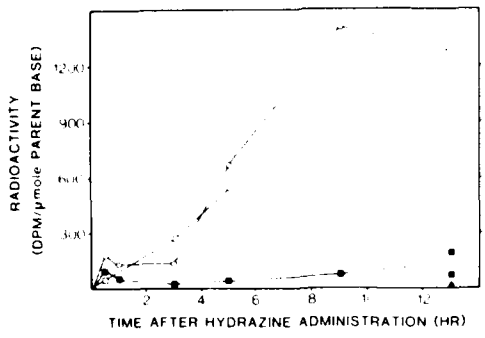

Figure 8. Variation with time in methylation of liver DNA in hydrazine-treated mice: 5-methylcytosine in control (open circles) and hydrazine-treated (open squares) mice, 7-methylguanine (closed squares) and O^6-methylguanine (closed triangle) in hydrazine-treated mice.

Groups of two rats and ten mice were given intraperitoneally 25 mg hydrocortisone (33.4 mg hydrocortisone sodium succinate) per kg body weight and immediately thereafter ^{3}H-methyl-methionine (100 μCi in 0.1 ml 0.9% NaCl for rats and 20 μCi in 0.03 ml saline for mice); methionine injections were repeated hourly until death. Animals were killed 0.5, 1, 3, and 5 hours, and also 9 hours for mice, following hydrocortisone treatment. No methylated guanines could be detected with satisfactory reproducibility in liver DNA prepared from these animals; Table 4 shows that little effect of hydrocortisone could be demonstrated on 5-methylcytosine production in rat liver DNA; similar measurements were not made in mouse liver DNA. Possibly, increased levels of circulating corticosteroids alone are not sufficient to produce aberrant DNA methylation and the presence of hydrazine may also be required. That question remains to be investigated.

Effect of Ethionine Pretreatment on DNA Methylation in Hydrazine-Poisoned Rats

The methionine analog, ethionine, can deplete S-adenosylmethionine pools and inhibit DNA methylase. Since the results of the studies on DNA methylation in hydrazine-treated animals suggest that the methylation process is mediated by S-adenosylmethionine and may be catalyzed by a DNA methylase, an experiment was carried out to determine whether ethionine could block DNA methylation in hydrazine-treated rats.

Two groups of two rats were given 300 μCi ^{3}H-methyl-methionine intraperitoneally to label the S-adenosylmethionine pools; 15 minutes later two rats were given 500 mg

TABLE 4

TIME-RESPONSE RELATIONSHIP FOR METHYLATION OF LIVER DNA IN RATS TREATED WITH HYDROCORTISONE

Hrs. Following Hydrocort. Adm.	5-Methylcytosine (dpm/ μmole cytosine)*	
	Control	Hydrocortisone
0.5	27	27
1.0	81	57
3.0	-	223
5.0	271	345

* no 7-methylguanine or O^6-methylguanine was detected.

ethionine/kg body weight intraperitoneally (saline was given to controls); 15 minutes later all rats were given 60 mg hydrazine/kg body weight and killed 3 hours later. Liver DNA was analyzed for methylated bases, and the results are summarized in Table 5.

TABLE 5

EFFECT OF ETHIONINE ON DNA METHYLATION IN HYDRAZINE-TREATED RATS

Treatment	Amt. Methylated Base, (μmoles methylated base/mole parent base)		
	7-Methylguanine	O^6-Methylguanine	5-Methylcytosine
Control	36	nd	33
Ethionine	nd	nd	159

nd = none detected

The inhibition by ethionine of the formation of 7-methylguanine in liver DNA is consistent with the known mechanism for the amino acid analog as an inhibitor of DNA methylase and supports a conclusion that the DNA methylation observed after hydrazine administration is dependent upon DNA methylase. The amount of 5-methylcytosine, however, seemed to be increased in the ethionine-pretreated animals; to be consistent with the hypothesis for the inhibition of DNA methylase, an additional mechanism must be proposed; for example, 5-methylcytosine is removed from liver DNA more slowly in ethionine-pretreated, hydrazine-poisoned rats. This hypothesis remains to be tested experimentally.

In a second experiment ethionine (500 mg/kg body weight) was given 12 hours before hydrazine treatment (60 mg/kg body weight orally). ^{3}H-Methyl-methionine was given at the same time as hydrazine and hourly thereafter until the rats were killed 5 hours after hydrazine administration. The liver DNA from these animals contained 112 µmoles 7-methylguanine per mole guanine and 188 µmoles 5-methylcytosine per mole cytosine; these levels are comparable to 28-58 µmoles 7-methylguanine per mole guanine and 88 µmoles 5-methylcytosine per mole cytosine in liver DNA from rats under similar conditions except without ethionine pretreatment. Apparently, ethionine, in order to block DNA methylation in hydrazine-treated rats, must be administered soon before hydrazine. Ethionine given 12 hours before hydrazine appears to increase the effect of hydrazine on DNA methylation; since ethionine is also hepatotoxic, the increased DNA methylation may reflect greater toxic insult to the liver due to the combination of hydrazine and ethionine.

Alkylation of Liver DNA in Rats Treated with Ethionine and Hydrazine

Large doses of ethionine given to rats result in the formation of small amounts of 7-ethylguanine in liver DNA, but small doses appear totally ineffective (Swann et. al., 1971). We decided to determine if ethionine could replace methionine in the alkylation of liver DNA in hydrazine-treated rats. Two rats were given intraperitoneally 25 µCi ethyl-1-^{14}C-L-ethionine (4.6 mCi/mmol; 100 µCi/3.54 mg; 0.89 mg ethionine/137-142 g body weight) and 60 mg hydrazine/kg body weight orally; an hour later rats were given a second injection of 25 µCi ethionine-^{14}C. The animals were killed 5 hours after receiving the hydrazine. Since ethionine is more persistent in liver than is methionine we felt that administration of hourly radiolabeled ethionine was unnecessary. The liver DNA contained no detectable 7-ethylguanine, a result which agrees with the literature for such low doses of ethionine, but it did contain 7 µmoles O^{6}-ethylguanine per mole guanine (quantitation is based on the assumption that the specific activity of O^{6}-ethylguanine is equal to that of the administered ethionine). While ethionine apparently does not replace methionine in the alkylation of DNA in hydrazine-treated rats, hydrazine toxicity seems to alter the ethylation of DNA by non-toxic doses of

ethionine. The relationship between ethionine and hydrazine will be studied further in an attempt to elucidate the mechanism by which hydrazine brings about methylation of liver DNA.

Methylation of Liver DNA in Rats Treated with Hepatotoxins Other than Hydrazine

Experiments with ethionine suggested that methylation of liver DNA in hydrazine-treated rats may not be specific for hydrazine but may be a general response to toxic liver injury. The effect of hepatotoxins other than hydrazine on methionine-mediated methylation of liver DNA was investigated.

Two unrelated hepatotoxins, carbon tetrachloride (CCl_4) and thioacetamide (C_2H_5NS), were given to rats in an experimental protocol similar to that used in the hydrazine study.

DNA Methylation in Carbon Tetrachloride-Treated Rats

Eighteen male Fischer 344 rats (190-330 g body weight) were given in corn oil orally 1 g carbon tetrachloride per kg body weight (LD_{50}) and killed 7 hours and 45 minutes later. Liver DNA was hydrolyzed in 0.1 M HCl at 70°C for 30 minutes. The hydrolysate was fractionated by reverse phase liquid chromatography under the following conditions: Whatman ODS-2 50 cm preparative column, water:methanol gradient of 10% to 100% methanol developed over 60 minutes at 4 ml/min; water adjusted to pH 4.00 with acetic acid; gradient curve N=2.5 concave. Fractions eluting where 7-methylguanine and O^6-methylguanine were expected were collected, evaporated to dryness, and redissolved in 0.1 M HCl. These acidic fractions were analyzed by fluorescence liquid chromatography using a strong cation exchange column. The original DNA hydrolysate from CCl_4-treated rats contained 58 μ moles O^6-methylguanine per mole guanine; no 7-methylguanine was detected (Figure 9).

The identity of O^6-methylguanine was confirmed in three tests:

1. O^6-methylguanine standard, added to the test hydrolysate, cochromatographed with the suspect material in cation exchange liquid chromatography.

2. Heating the test hydrolysate in 1.0 M HCl at 100°C for 1 hour almost completely destroyed the suspect O^6-methylguanine content but increased the guanine content; this would be expected in the acid hydrolysis conversion of O^6-methylguanine to guanine.

3. The fluorescent O^6-methylguanine chromatographic elution peak disappeared when fluorescence excitation wavelength was changed from 286 nm (excitation maximum for O^6-methylguanine) to 253 $\pm$ 1 nm (excitation minimum for O^6-methylguanine).

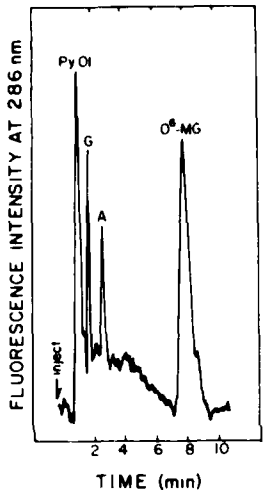

Figure 9. Elution profile of partially purified chromatographic fraction of liver DNA hydrolysate prepared from a carbon tetrachloride-treated rat; Py. Ol., pyrimidine oligonucleotides; G, guanine, A, adenine, and O^6-MG, O^6-methylguanine.

Methionine-Mediated DNA Methylation in Thioacetamide-Treated Rats

Three rats (male Fischer 344, 130-203 g body weight) were given 200 mg thioacetamide/kg body weight (LD_{50}) orally in water and 100 µCi ^{3}H-methyl-methionine intraperitoneally; the methionine injections were repeated hourly until the rats were killed 5 hours after thioacetamide administration. Liver DNA was isolated and fractionated into its component pyrimidine oligonucleotides and purine bases. Each fraction was evaporated to dryness in its individual round bottom flask and recovered in 2 ml 0.1 M HCl which was assayed for radioactivity by liquid scintillation. Hydrolysate from thioacetamide-treated rats contained an estimated 27 µmoles of 7-methylguanine per mole guanine but only a possible trace of O^6-methylguanine.

These studies are being expanded to include several other hepatotoxins.

Histopathologic Analysis of Liver After Hydrazine Poisoning

The literature is lacking a detailed description of the histopathologic changes that take place in the liver of rats given orally various doses of hydrazine. Since it is felt that the DNA methylation seen in livers of hydrazine-poisoned animals may be indicative of a non-specific response of the liver to hepatotoxins, it is imperative to describe the extent of liver damage seen in hydrazine-treated animals at the doses used in this project.

Twelve male Fischer 344 rats (205-265 g) were fasted overnight and given by stomach tube 60 mg hydrazine/kg body weight in 0.1 M HCl. Two hydrazine-treated rats and two control rats (given only 0.1 M HCl) were killed 5 minutes later and at 1, 5, 9, 12, and 24 hours after treatment. A section from each liver was placed in 10% neutral buffered formalin and slices were prepared for hematoxylin-eosin staining.

A preliminary reading of the slides indicates a rapid, progressive formation of fat accumulation in hepatocytes with necrosis apparent 9 hours after dosing. The study will be repeated with more doses and using lipid-specific staining techniques.

Effect of Hydrazine on In Vitro Methylation of DNA

An in vitro system for measuring DNA methylation quantitatively was developed in an effort to determine whether liver DNA methylation occurs in human tissue treated with hydrazine. A rat liver homogenate was provided with radiolabeled S-adenosylmethionine, a NADPH-generating system, and DNA from a variety of sources and incubated with hydrazine.

Fresh liver was homogenized in an equal volume of 1.15% KCl in 0.2 M sodium/potassium phosphate pH 7.7. One milliliter of homogenate was incubated for 5 minutes at 37°C with added DNA and a NADPH-generating system of 0.50 mM NADP, 1.67 mM glucose-6-phosphate, 0.10 mM nicotinamide, 5.0 mM magnesium chloride in 4.0 ml 0.2 M sodium/potassium phosphate pH 7.4. ^{3}H-Methyl-S-adenosylmethionine (10 µCi in 0.10 ml HCl pH 3.0) and hydrazine (0.60 µl in 0.10 ml HCl pH 3.0; equivalent to 1.88 mmoles hydrazine/kg body weight) were added with 0.80 ml phosphate buffer. The reaction mixture was shaken vigorously for 30 minutes in a 37°C water bath, and the reaction was stopped by plunging the incubates into ice and adding 1 ml of 3.5% naphthalene-1,5-disulfonic acid. DNA was recovered and purified by the standard method of Swann and Magee (1968).

The results are summarized in Table 6. The amounts of methylation obtained varied with the source of DNA, which was expected, as this reflects the variability of

methylation site availability in different DNA molecules. The extent of 5-methylcytosine obtained was approximately the same as reported in the literature for purified DNA methylase preparations (Morris and Pih, 1971). Aberrant methylation of DNA guanine was detected in this in vitro system. B. subtilis DNA appears to be a particularly interesting substrate in that the formation of 7-methylguanine occurred in the absence of hydrazine, and when hydrazine was added to boiled homogenate (inactivated DNA methylase) and B. subtilis DNA, the amount of 7-methylguanine increased. These studies will be continued and extended to include human liver preparations.

TABLE 6

IN VITRO METHYLATION OF DNA WITH RAT LIVER HOMOGENATE AND HYDRAZINE

DNA Substrate	Hydrazine Conc. (mM)	Methylated Bases (nmoles/mole parent base)*	
		5-Methylcytosine**	7-Methylguanine
rat liver naked	1.88	113	54
rat liver naked	1.88	428	28
rat liver chromatin	1.88	35	n.d.
salmon testes naked	1.88	n.d.	n.d.
B. subtilis naked	0	1233	144
B. subtilis*** naked	1.88	351	279

* no O^6-methylguanine was detected; n.d. = none detected

** for B. subtilis DNA, determination was done for total pyrimidine oligonucleotides, not isolated 5-methylcytosine

*** liver homogenate was boiled before incubation

MONOMETHYLHYDRAZINE (MMH) METABOLISM

DNA Methylation in MMH-Treated Rats

MMH is a weak methylating intermediate of liver DNA in rodents (Hawks and Magee, 1974). It is a possible intermediate in DNA methylation in hydrazine-poisoned animals. If MMH is an effective intermediate in the hydrazine-stimulated DNA methylation process, then administration of MMH in lieu of hydrazine should also produce sufficient methylated bases to permit detection by the techniques used for the hydrazine studies.

Two rats were intubated with 15 mg ^{3}H-methyl-monomethylhydrazine (0.33 mmole) per kg body weight (1.5 mg mCi per kg body weight in 0.96 ml 0.1 M HCl; approximately 190 μCi per rat). Animals were killed 5 hours after MMH administration and liver DNA was analyzed for methylated guanines. The 7-methylguanine detected in two trials of this experiment amounted to 5 and 21 μmoles/mole guanine analyzed. This was calculated from the radioactivity cochromatographing with authentic 7-methylguanine and assuming the specific activity of the methylated purine was the same as that of the administered MMH. No O^{6}-methylguanine was detected.

DNA Methylation in MMH-Treated Mice

Twelve mice were intubated with 14.4 mg ^{3}H-methyl-monomethylhydrazine (equimolar to 10.0 mg (0.33 mmole) hydrazine per kg body weight), 28.3 μCi per animal in 0.1 ml 0.1 M HCl. The mice were killed 5 hours after MMH administration and liver DNA was analyzed for methylated guanines. Liver DNA from these mice contained 338 μmoles 7-methylguanine per mole guanine, but no O^{6}-methylguanine could be detected. Quantitation of the 7-methylguanine assumed the specific activity of the methylated base was equal to that of the administered MMH.

It is possible in the case of the mouse to compare the extent of 7-methylguanine formation in liver DNA following hydrazine and MMH administration, as the two toxicants were studied at equimolar doses. Administration of 0.33 mmole MMH/kg body weight resulted 5 hours later in 388 μmoles 7-methylguanine/mole guanine, compared to 73-142 μmoles in DNA from mice treated with an equimolar dose of hydrazine.

The studies in the rat indicated that the amount of DNA methylation in hydrazine-treated animals was underestimated because of the difficulty in quantitating the specific activity of the S-adenosylmethionine pool. If the measurement of 7-methylguanine in

mouse liver DNA 5 hours after hydrazine administration were compensated for the underestimation of methylated bases, then there apparently would be no real difference in DNA methylation levels at 5 hours in hydrazine- and MMH-treated mice. Again, as in the studies using the rat, the amount of DNA methylation observed in the MMH-treated mouse is far less than would be expected by extrapolation from the hydrazine experiments if MMH served more proximal in the DNA methylation pathway in hydrazine-treated animals.

1,1-DIMETHYLHYDRAZINE (UDMH) METABOLISM

Since UDMH is also suspected of being carcinogenic, its role in forming adducts with DNA was also investigated. One Fischer 344 male rat, 318 g body weight, was given orally 1.91 mg UDMH/kg body weight (^{14}C-UDMH, 13.3 mCi mmole; ^{3}H-UDMH, 4 Ci/mmole; prepared in the laboratory). A second rat, 369 g body weight, was given the same dose of UDMH not radioactively labeled. Both animals were killed 5 hours after UDMH administration and lungs, colons, and kidneys were pooled for DNA isolation; DNA was isolated from individual livers.

Liver DNA isolated from the radioactive animal weighed 15.4 mg and from the non-radioactive animal, 23.3 mg. DNA from pooled tissues weighed 4.0 mg from colon, 11.0 mg from kidney, and 2.2 mg from lung. The DNA samples were hydrolyzed in 0.1 M HCl for 40 minutes at 70°C, and the hydrolysates were filtered (0.65 μ filter pore size). Aliquots (100 μl, 500 μg DNA) were counted for radioactivity and the remainder of each hydrolysate was fractionated by high pressure liquid chromatography. The total radioactivity in the DNA was 8 cpm/mg liver DNA, 45 cpm/mg kidney DNA and 78 cpm/mg lung DNA. These are virtually insignificant amounts of radioactivity associated with unfractionated DNA hydrolysates; nevertheless, the hydrolysates containing added 7-methylguanine and O^6-methylguanine as carrier were fractionated to determine if all the radioactivity could be associated with one particular base. The results of the radioanalysis of the DNA fractions are summarized in Table 7.

Except for liver pyrimidine oligonucleotides, the fractions listed in Table 7 have insignificant amounts of radioactivity; as shown in earlier studies (Shank, 1979) the methyl groups of UDMH contribute to the one-carbon pool in liver and thus labeling in the liver pyrimidine oligonucleotides may represent normal synthesis of 5-methylcytosine. The reverse of the ^{3}H/^{14}C ratio observed in kidney O^6-methylguanine is unexpected. It is unlikely that the radioactivity in the kidney DNA O^6-methylguanine fraction represents contamination from adenine because of the greater amount of activity in the O^6-methylguanine fraction and the excellent resolution between the two peaks.

TABLE 7

INCORPORATION OF RADIOLABEL FROM (^{3}H,^{14}C) METHYL-1,1-DIMETHYLHYDRAZINE INTO DNA OF RATS

Tissue	Amt. DNA	DNA Fraction	Total dpm in Fraction ^{3}H	Total dpm in Fraction ^{14}C
Liver	15.4	pyrimidine oligo	364	166
Liver	15.4	guanine	11	6
Liver	15.4	7-methylguanine	20	7
Kidney	11.0	adenine	25	20
Kidney	11.0	O^{6}-methylguanine	75	141
All Others	-	all		no counts

SUMMARY AND CONCLUSIONS

Studies on the methylation of liver DNA in animals exposed to hydrazine have been extended toward characterization of the mechanism of action of this unusual response. Two possible mechanisms were proposed (Shank, 1979): one, that hydrazine could be methylated in vivo to MMH which is a known, weak, indirect methylating agent (Hawks and Magee, 1974), and two, hydrazine may stimulate aberrant activity of endogenous DNA methylase.

Methylation of rat and mouse liver DNA was only weakly dependent upon the amount of hydrazine administered, for the dose range studied (from below the estimated $LD_{0.01}$ up to the LD_{50}). Five hours after hydrazine administration the production of 5-methylcytosine, the only methylated base in normal mammalian DNA, was unaltered in all animals except mice receiving the highest dose of toxicant. The formation of 7-methylguanine was rapid, occurring within one-half hour following hydrazine administration and could be stimulated for several hours; the amount of 7-methylguanine in liver DNA did not increase greatly with time, however. Since the protocol required repeated administration of radiolabeled methionine to keep the S-adenosylmethionine pool labeled, it is possible that hydrazine administration may produce a rapid increase in the number of methylated sites available in DNA, rather than acting on the methylase system; giving animals more hydrazine would not necessarily increase the number of these sites, and once the sites are made available they may be methylated rapidly so that the final number of sites methylated is not strongly dependent upon time. One rat study

indicated that liver DNA was no longer methylated at the 7-position of guanine if the injections of radiolabeled methionine were delayed 19 hours after hydrazine administration. This observation might be interpreted as evidence that the increased availability of methylation sites in hydrazine-treated animals is a short-lived response.

Methylation of the O^6-position of guanine was detectable nine hours after hydrazine administration. The delay for this methylation is not explained; however, the levels of O^6-methylguanine may parallel the levels of 7-methylguanine, yet not reach the limit of detection in the chromatography system until nine hours after poisoning. Alternatively, the methylation of guanine at the two sites may proceed by separate mechanisms.

Fluorescence spectrophotometric analysis has confirmed the presence of 7-methylguanine and O^6-methylguanine in liver DNA from hydrazine-treated animals. The amounts of these bases were approximately two orders of magnitude below corresponding levels for a toxic dose of a strong methylating intermediate such as dimethylnitrosamine or 1,2-dimethylhydrazine but were similar to levels seen following treatment with carbon tetrachloride and thioacetamide. The fact that aberrant DNA methylation can be demonstrated with three structurally unrelated hepatotoxins (and hepatocarcinogens) suggests that the methylation may be more a response to toxic insult to the liver rather than a response specifically characteristic to hydrazine.

Sufficient evidence seems available now to reject the hypothesis that MMH is an important intermediate in hydrazine-stimulated DNA methylation. The amounts of 7-methylguanine observed in MMH-treated rats and mice were less than expected for a more proximal intermediate in the methylation pathway. Dost (1979) has recently shown that 75% of a dose of 1 mmole hydrazine per kg body weight was excreted by rats within 48 hours as expired nitrogen gas and as hydrazine or hydrazine conjugate(s) in the urine. Less than 100% of administered hydrazine, then, would be available for conversion to MMH in vivo. The time-response studies indicate that much of the DNA methylation can occur within 30 minutes of hydrazine administration, and the dose-response studies indicate little dependency upon the amount of hydrazine administered and the extent of methylation. None of the studies, then, comparing DNA methylation following administration of hydrazine or MMH supports the hypothesis that MMH is an intermediate in DNA-methylation in hydrazine-treated animals.

The results with carbon tetrachloride and thioacetamide suggest that the hydrazine effect on DNA methylation could be a response to toxic stress. Ruchirawat (1974) demonstrated a similar methionine-dependent methylation of liver DNA in response to a hepatotoxic dose (30 mg/kg body weight) of dimethylnitrosamine. Also, hydrocortisone has been reported (Kudryashova and Vanyushin, 1976) to stimulate methylation of DNA

cytosine. Hence, it was proposed (Shank 1979) that hepatotoxicity itself may stimulate a corticosteroid-mediated methylation of DNA. The work of Kudryashova and Vanyushin (1976) indicated that hydrocortisone increased liver DNA cytosine methylation by increasing the activity of DNA methylase and by increasing the number of methylation sites available. Our hydrocortisone experiments failed to show a significant increase in DNA cytosine methylation or to detect any methylation of DNA guanine, but these studies did not look at DNA methylation in animals exposed to both hydrocortisone and hydrazine, thus little can be concluded from the data at hand.

The effect of early pretreatment of rats with ethionine before hydrazine administration was particularly interesting. Ethionine, a well-known hepatotoxin, is thought to serve as an adenosine sink, thus depleting S-adenosylmethionine pools, among others; early pretreatment of rats with ethionine, however, appeared to increase the methylation of DNA cytosine and guanine in hydrazine-treated rats. Also, ethylation of the O^6-position of guanine in DNA was demonstrable in a hydrazine-treated rat given a non-toxic dose of ^{14}C-ethyl-ethionine, whereas other laboratories have not detected this ethylation in rats given only ethionine at the LD_{50} level. Such observations are compatible with the hypothesis that hepatotoxins may produce a rapid increase in the availability of methylation sites in liver DNA. It is possible that such a mechanism evolved to alter DNA function during hepatotoxicity to improve chances of survival for hepatocytes.

The carcinogenicity of hydrazine may be causally related to the toxicity of the compound rather than to some intrinsic property of the toxicant. The covalent binding of reactive species to DNA has been related to both toxicity and carcinogenicity (see Lawley 1976), but these hydrazine studies offer the first evidence that toxicity itself may be the stimulus of the initiating event (DNA methylation) in carcinogenesis, rather than merely stimulating turnover of cells already genetically damaged. This may be particularly relevant to instances in which chemical agents induce tumor formation only at toxic exposures, such as with hydrazine.

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PART II. NAPHTHALENE TOXICITY AND METABOLISM

INTRODUCTION

OVERALL AIMS

A new laboratory has been established in the subprogram on Biochemistry and Metabolism which will focus its efforts on pulmonary toxic agents which require metabolic activation. Studies on Air Force related materials will be used not only to gain insights into the mechanisms and biochemical consequences of chemically-induced lung damage but also to develop new, sensitive methods of detection of such damage in animals. This research program will approach several basic questions about toxic injury to the lung including: the roles and the interrelationships of various pulmonary xenobiotic toxifying and detoxifying enzymes, the role of hepatic metabolism in the formation of proximate or ultimate lung toxic metabolites, and the mechanisms of tolerance and alterations in pulmonary biochemistry caused by repeated exposure to such pulmonary toxins. Once a clear understanding of the metabolic steps critical to the lung toxicity of a particular compound in animals species has been attained and appropriate methods have been developed for studying these processes in vitro, the investigations will be extended to include fresh human lung autopsy tissue.

Such research is relevant to Air Force interests from several standpoints and these will be discussed throughout the Introduction. The pulmonary toxicant currently under study is the hydrocarbon naphthalene, of direct interest to the Air Force because of its presence in and structural similarity to many of the hydrocarbons in shale oil (Guerin et

al., 1978). Moreover, naphthalene is a starting material in the synthesis of two Air Force materials, Decalin and tetrahydronaphthalene. In mice, intraperitoneal doses of this hydrocarbon cause necrosis of the non-ciliated bronchiolar epithelial (Clara) cells (Mahavi et al., 1977), cells which have been identified as a major locus of pulmonary cytochrome P-450 mixed function oxygenase activity (Boyd, 1977; Boyd et al., 1978; Serabjit-Singh et al., 1980; Devereux et al., 1979) and which may be important target cells in the cytotoxicity and carcinogenicity of chemicals which require metabolic activation. Because of the potential role of this cell type in the pathogenesis of lung diseases such as cancer, emphysema and fibrosis which may arise from exposure to environmental chemicals, a knowledge of factors which affect the metabolic functioning of this cell type in the lung is essential. Many of the materials of concern to the Air Force are inhaled agents which may cause subtle alterations in the functioning of cells in the lung that are discernible biochemically but not pathologically. Thus, the elucidation of the metabolic steps critical to the pulmonary toxicity of naphthalene may yield a sensitive method for determining whether inhalation exposure to other Air Force compounds such as the hydrazines or jet fuels affects the metabolic functions of cells like the Clara cell. Modification of the rates at which such metabolic processes occur in lung tissue would certainly have profound implications in situations where personnel are exposed to both work related compounds and chemicals like those in cigarette smoke.

Another question of considerable importance to the Air Force is whether animal species currently in use in the toxicity studies at THRU are appropriate models for the human. The mouse appears to be more sensitive to lung damage by a variety of metabolically activated xenobiotics than other rodent species. For example, systemic administration of 3-methylfuran (Boyd et al., 1978), butylated hydroxytoluene (Adamson et al., 1977), bromobenzene (Reid et al., 1973) and naphthalene (Reid et al., 1973) causes pulmonary damage in the mouse but not in the rat. Likewise, equivalent doses of carbon tetrachloride cause more pronounced damage in mouse lung than in rat lung (Boyd et al., 1980). A further example of species specific lung damage is that of 3-methylindole, a metabolically activated toxin which damages the lungs of cattle, sheep and goats but not rodents (Bray and Carlson, 1979; Hammond et al., 1980). Potential explanations of these differences are that the substrate specificities of the pulmonary cytochrome P-450 monooxygenases differ between species or that enzymes responsible for the detoxification of the "toxic" metabolite(s) are not active in sensitive but are active in resistant species. While rodent lung xenobiotic metabolizing enzymes have been the subject of numerous studies, little work has focused on the characterization of these enzymes in human lung tissue (Prough et al., 1977; Prough et al., 1979; McManus et al., 1980). Thus one of the goals of the research in this subprogram is to develop appropriate in vitro methods for studying the rates at which critical metabolic steps in the activation and detoxification of a compound like naphthalene are undertaken in animal lung tissue so that such processes can be more fully characterized in human lung tissue.

BACKGROUND

Naphthalene Lung Toxicity

Reid et al. (1973) were the first to report that intraperitoneal administration of 350 mg/kg naphthalene reproducibly elicited bronchiolar and bronchialar epithelial cell necrosis in mice. Mahavi and coworkers (1977) further characterized this toxicity using both light and electron microscopic techniques. These authors observed that naphthalene induced selective damage to Clara cells which was both time and dose-dependent. Maximal necrosis was evident 12 to 24 hours after hydrocarbon administration; lesions were resolved 7 days after exposure. Lung damage was extensive after doses of 256 mg/kg and only marginal after doses of 64 mg/kg.

Naphthalene Metabolism

The metabolic pathways for naphthalene have been studied thoroughly both in vivo (Corner and Young, 1954; Boyland et al., 1961) and in vitro (Jerina et al., 1970; Oesch and Daly, 1970). The overall pathways are thought to include the formation of the relatively stable naphthalene-1,2-epoxide (t = 4 min) followed by glutathione conjugation, non-enzymatic rearrangement to form 1-naphthol or hydration to form a dihydrodiol (epoxide hydrase). Phenols and diol metabolites of naphthalene are known to form glucuronide and sulfate conjugates. More recent investigations have shown that naphthalene is metabolized in the rat to yield a series of urinary metabolites which may be derived from epoxide, dihydrodiol epoxide and diepoxide precursors (Stillwell et al., 1978; Stillwell et al., 1979; Stillwell et al., 1980). Not unexpectedly, both the dihydrodiol epoxide and the diepoxide metabolites are considerably more toxic in both the rat and the mouse than the parent compound. The nature of this toxicity was not reported. By analogy, the findings that the 7,8-dihydrodiol-9,10-epoxide metabolite of benzo(a)pyrene (Weinstein et al., 1976) is a major DNA binding metabolite and is perhaps the ultimate carcinogen formed from this polycyclic aromatic hydrocarbon suggest that the dihydrodiol epoxide and diepoxide metabolites of naphthalene may be biologically quite important. A recently reported study (Hesse and Mezger, 1979) has shown that rat liver microsomal metabolism of ^{14}C-naphthalene results in the formation of reactive metabolites which bind covalently to the microsomal protein. A major portion of the bound radioactivity appears to arise from the secondary metabolism of 1-naphthol and not directly from the parent hydrocarbon itself. Whether the formation of one or more of these naphthalene metabolites plays a role in the pulmonary toxicity of the parent hydrocarbon in mice remains to be determined.

RESEARCH PROGRAM

ASSESSMENT OF TISSUE DAMAGE

Initial studies were conducted to confirm the earlier work of Mahavi et al. (1977) (done in C57/BL6 mice) in the Swiss Webster mouse (the strain to be used for the biochemical studies) and to determine whether naphthalene caused necrotic lesions in other tissues such as the kidney or liver which also contain cytochrome P-450 monooxygenase activity. A further aim of this experiment was to determine whether pretreatments which alter potential pathways of naphthalene toxication (such as metabolism by the cytochrome P-450 MFO) or detoxification (through glutathione conjugate formation) would enhance or inhibit the pulmonary toxicity of naphthalene.

Male Swiss Webster mice (20-25 g) were purchased from Simonsen Breeding Laboratories, Gilroy, California, and were housed in the animal vivarium at UCI for at least 5 days prior to use. Animals were allowed food and water ad libitum. Mice were divided into groups of 5 each and were given intraperitoneal injections of either vehicle, the cytochrome P-450 MFO inhibitor piperonyl butoxide (1600 mg/kg, Chemical Dynamics Corporation, South Plainfield, New Jersey) or the glutathione depleting agent, diethyl maleate (600 µl/kg, Aldrich Chemical Company, Milwaukee, Wisconsin). Naphthalene was administered intraperitoneally 30 minutes later at the dose indicated in Table 1. All compounds were dissolved in corn oil such that 1 cc was given per 100 g body weight.

Those animals surviving 24 hours were sacrified with an overdose of pentobarbital. The lungs and heart were removed en bloc by exposing and ligating the trachea and then by carefully dissecting away both organs. Three millimeter sections of liver and both kidneys were fixed with the lungs in 10% buffered formalin. Tissues were embedded in paraffin, cut in 5-6 µ sections and stained with hematoxylin and eosin.

Data on the mortality and site of tissue necrosis 24 hours after the administration of naphthalene or naphthalene plus piperonyl butoxide or diethyl maleate are presented in Table 1. Photomicrographs showing terminal bronchiolar airways of naphthalene-treated mice with or without diethyl maleate or piperonyl butoxide pretreatment are shown in Figure 1A-1F.

In the most severely damaged lungs, there was extensive necrosis of the bronchial and bronchiolar epithelial cells, and large numbers of exfoliated cells were observed in the bronchiolar lumen. In less severely damaged lungs, the normal cuboidal appearance of the bronchiolar epithelium was disrupted and a few cells, especially in the smaller airways, were detached from the basement membrane. The epithelial lining of larger airways appeared less sensitive than that of the smaller airways. Paraffin embedded 5 µ sections would not allow differentiation of damage to ciliated vs non-ciliated cells.

TABLE 1

PERCENT MORTALITY AND SITE OF TISSUE NECROSIS
24 HOURS AFTER INTRAPERITONEAL ADMINISTRATION
OF NAPHTHALENE IN MICE

Pretreatment	Dose of Naphthalene (mg/kg)	Mortality(%)	Site of Tissue Necrosis Lung	Liver	Kidney
Corn oil	500	100	-	-	-
Corn oil	400	100	-	-	-
Corn oil	300	20	+	0	0
Corn oil	200	60	+	0	0
Corn oil	100	0	+	0	0
Corn oil	50	0	0	0	0
Corn oil	0	0	0	0	0
Diethyl maleate	500	100	-	-	-
Diethyl maleate	300	100	-	-	-
Diethyl maleate	50	0	+	0	0
Diethyl maleate	0	0	0	0	0
Piperonyl butoxide	500	0	0	0	0
Piperonyl butoxide	300	0	0	0	0
Piperonyl butoxide	50	0	0	0	0
Piperonyl butoxide	0	0	0	0	0

This experiment demonstrated that naphthalene caused extensive dose-dependent damage to the bronchiolar epithelium. Later studies indicated that death due to naphthalene occurred in the first 12 to 24 hours; those animals surviving the first 24 hours have been kept for up to seven days with no further deaths. The 24 hour LD_{50}, calculated by the method of Litchfield and Wilcoxin (1949) is 380 mg/kg (350-413 mg/kg 95% C.L., n=65).

Pretreatment of mice with either diethyl maleate or piperonyl butoxide markedly altered both the mortality and the lung toxicity caused by a subsequent dose of naphthalene. None of the diethyl maleate pretreated mice given 300 mg/kg naphthalene survived 24 hours. Lungs of diethyl maleate pretreated mice given a subsequent 50

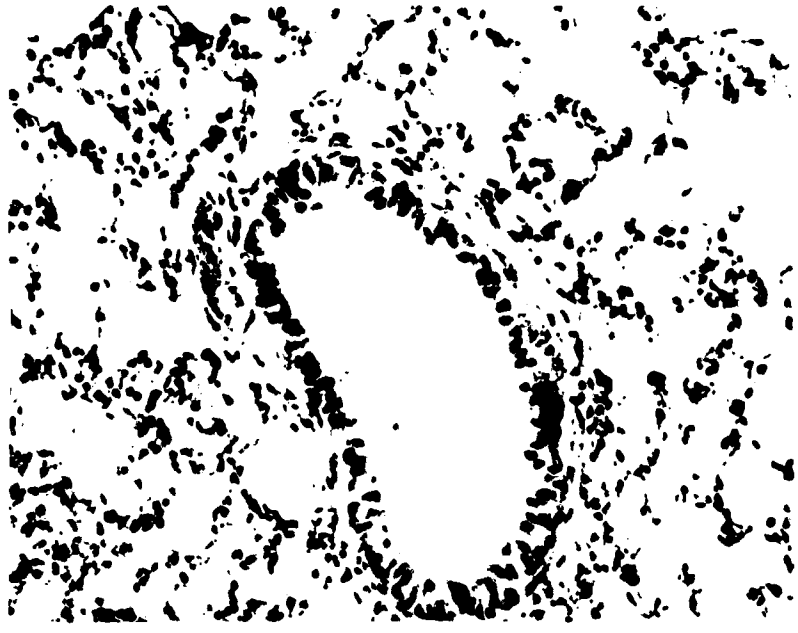


Figure 1A

Figures 1A-1F. Light micrographs of terminal bronchioles from mice sacrificed 24 hours after the intraperitoneal administration of A. corn oil; B. 50 mg/kg naphthalene; C. 100 mg/kg naphthalene; D. 200 mg/kg naphthalene; E. diethyl maleate plus 50 mg/kg naphthalene; and F. piperonyl butoxide plus 500 mg/kg naphthalene. (X200)

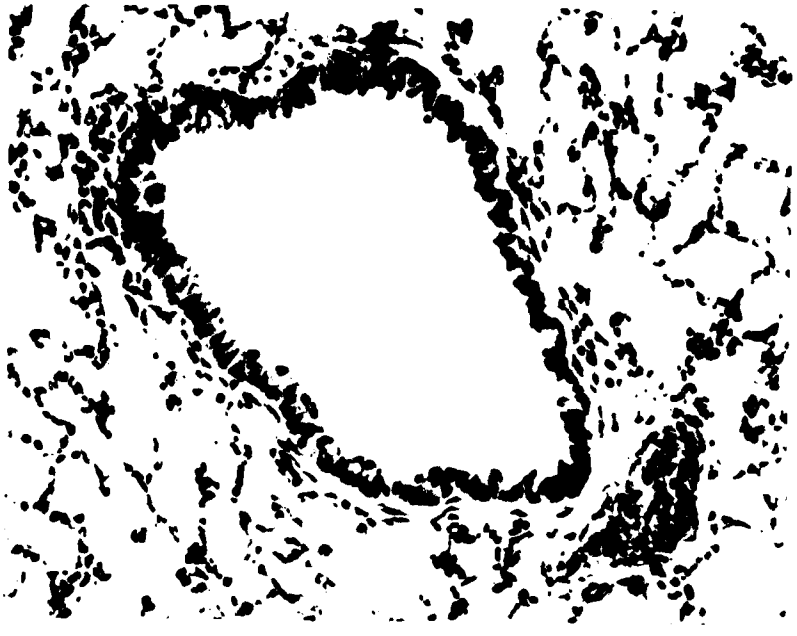


Figure 1B.

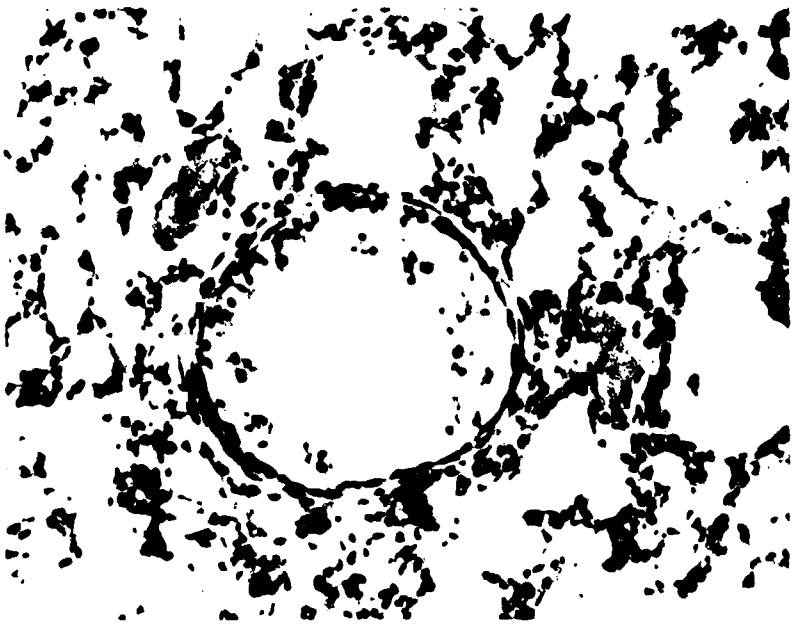


Figure 1C

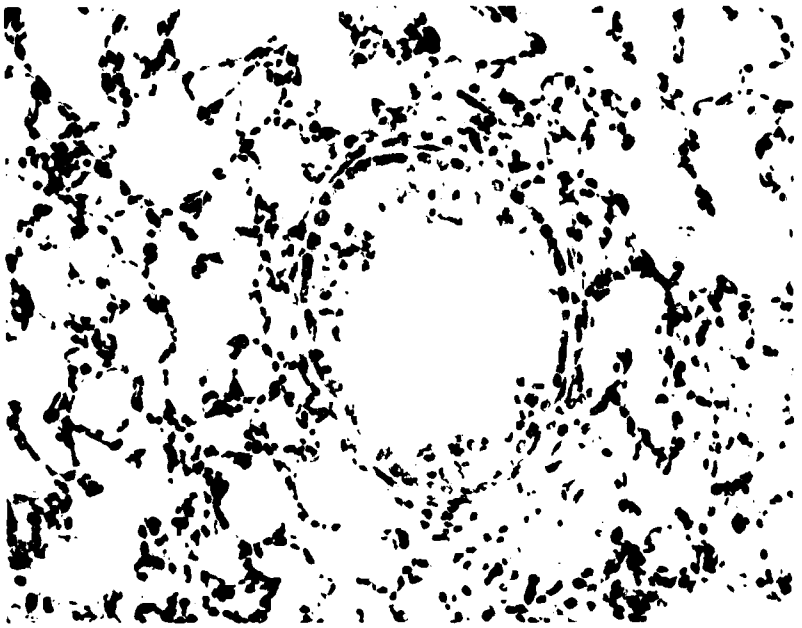


Figure 1D

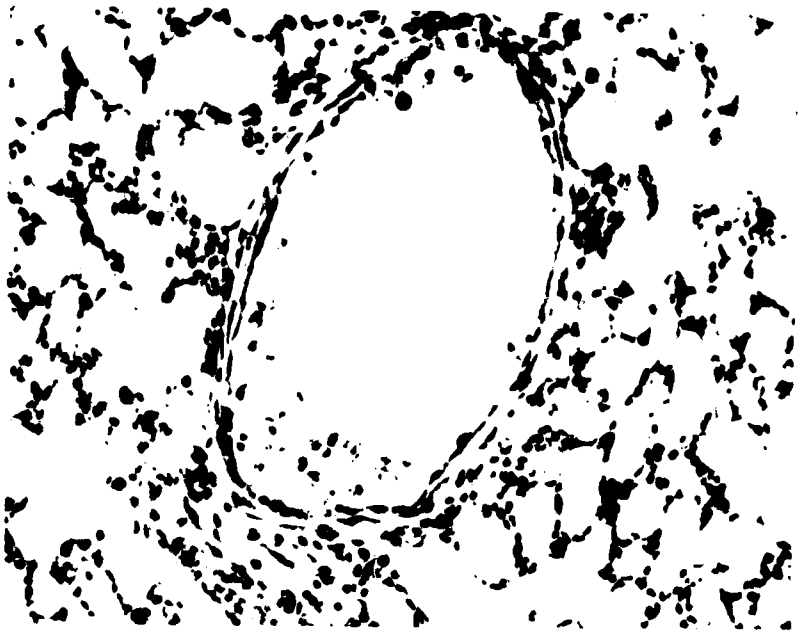


Figure 1E

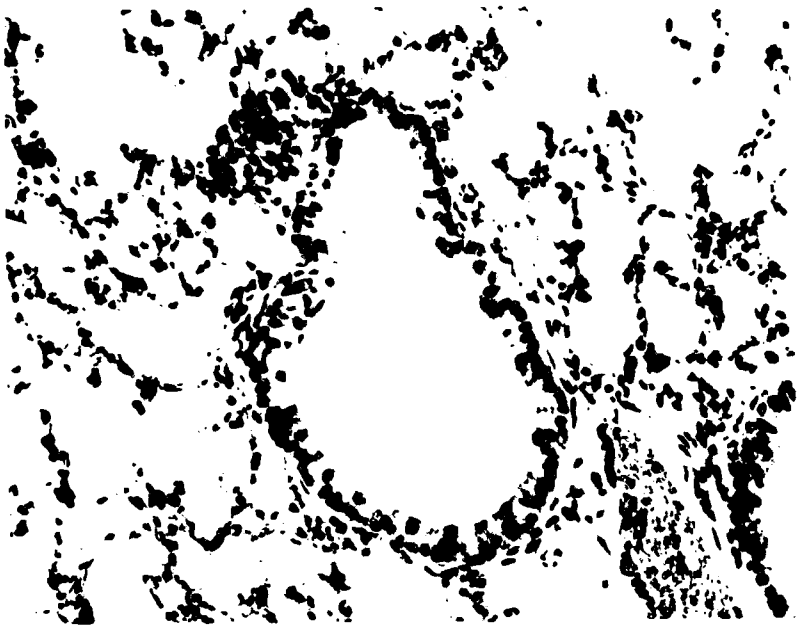


Figure 1F

mg/kg dose of naphthalene were severely damaged (Figure 1B, corn oil and 50 mg/kg naphthalene vs. Figure 1E, diethyl maleate and 50 mg/kg naphthalene). In contrast piperonyl butoxide pretreatment decreased the toxicity of naphthalene. All of the animals from the 500 mg/kg and 300 mg/kg naphthalene treatment groups pretreated with piperonyl butoxide survived 24 hours. Moreover, comparison of the photomicrographs of lung sections taken from mice treated with piperonyl butoxide and 500 mg/kg naphthalene with lung sections from control mice revealed few differences (Figure 1A vs Figure 1F). Cells lining some of the smallest airways from mice treated with piperonyl butoxide and 500 mg/kg naphthalene occasionally showed some ballooning

but the damage was slight. No exfoliated cells were observed in the lumen of any of the airways examined.

The results of this experiment provide a preliminary indication that the lung toxicity of naphthalene is due to metabolism by the cytochrome P-450 mixed function oxygenases in vivo and that tissue reduced glutathione levels play an important role in modulating the toxicity.

COVALENT BINDING OF REACTIVE METABOLITES AND RELATIONSHIP TO TOXICITY

Several reports over the past 5 years have demonstrated a close correlation between the covalent binding of electrophilic metabolites formed during the mixed function oxygenase catalyzed metabolic activation of a variety of xenobiotics and the cytotoxicity caused by these compounds. Examples of toxins where there is a close relationship between covalent binding and necrosis are the hepatotoxins bromobenzene and acetaminophen (Mitchell et al., 1976) and the pulmonary toxins butylated hydroxytoluene (Kehrer and Witschi, 1979) and 4-ipomeanol (Boyd and Burka, 1978). Studies on the covalent binding of a particular xenobiotic cannot be used to predict a priori that the compound will be cytotoxic. However, in instances where there is a close correlation between tissue damage and covalent binding, such data have been extremely useful in examining various factors which affect the formation and fate of metabolites which are too chemically reactive to be isolable from biological material. The results to be presented in the remainder of this report show that naphthalene is metabolized in vivo to metabolites which are sufficiently chemically reactive to bind covalently to tissue macromolecules and that this covalent binding is time and tissue-dependent.

COVALENT BINDING OF NAPHTHALENE METABOLITES IN VIVO

1-^{14}C-Naphthalene (5 mCi/mmole) was purchased from Amersham Searle, Arlington Heights, Illinois. The chemical and radiochemical purity was shown to be greater than 99.5% by high pressure liquid chromatography on a C$_{18}$ μBondapak Column in 65% methanol/35% H$_2$O at 1.0 ml/min. Fractions of the column eluate were collected every 30 seconds for liquid scintillation counting. Naphthalene eluted from the column at 9.5 minutes.

To determine whether naphthalene metabolites were bound to tissue macromolecules in vivo, two mice were given 200 mg/kg (676 dpm/nmole) doses of ^{14}C-naphthalene intraperitoneally and both animals were sacrificed by decapitation four hours later. Lung, liver, kidney and muscle were removed and homogenized in 3 volumes of distilled water. A 1 ml fraction of the homogenate was transferred to a conical

centrifuge tube containing an equal volume of 10% trichloroacetic acid. Unbound metabolites were removed by exhaustive solvent extraction of the precipitated macromolecules with ethanol/H_2O (70:30) until no further radioactivity could be removed. The precipitated fraction was then dissolved in 1 N NaOH, an aliquot was taken for protein determination by the method of Lowry et al. (1951) and a further aliquot was counted in 5 ml ACS (Amersham/Searle) in a Beckman 3150T liquid scintillation counter for 20 minutes. All counts were corrected for quench by internal standardization. The results of this experiment showed that radioactivity remaining in the macromolecular pellet was highest in the lung, liver and kidney and was much lower in muscle. The experiment also showed that use of specific activities of approximately 200 dpm/nmole would yield sufficient radioactivity in the final washed pellet for accurate determinations of bound naphthalene metabolites.

Two experiments were conducted to show that radioactivity associated with the washed pellet is bound covalently. In the first an aliquot of the NaOH digest (liver) was spotted on a TLC plate (Silica Gel G 250 μ) and the plate was successively run in chloroform, ethyl acetate and methanol. (Solvent was allowed to evaporate between each step.) Sections of the plate (0.5 cm) were scraped into liquid scintillation vials and radioactivity was determined in each fraction. All of the radioactivity remained with the protein at the origin.

The remaining NaOH protein digest from the livers was combined and sufficient 10% trichloroacetic acid was added to precipitate the tissue macromolecules. After centrifugation, the supernatant was discarded and the precipitate was redissolved in 1 N NaOH. After an aliquot of this digest was taken for determination of specific activity (cpm/mg protein), the process of precipitation and redissolution was repeated 3 more times. The specific activity of the digest remained constant ($X \pm$ S.E. = 62.7 ± 2.4 cpm/mg protein) during this process.

TISSUE DISTRIBUTION OF COVALENTLY BOUND NAPHTHALENE METABOLITES AND EFFECT OF DIETHYL MALEATE PRETREATMENT

To determine which tissues contain the largest amounts of covalently bound naphthalene metabolite(s) and whether pretreatment with diethyl maleate affects the binding of such metabolites, groups of 4 mice each were given corn oil or diethyl maleate (600 μl/kg in corn oil) intraperitoneally followed 30 minutes later by ^{14}C-naphthalene (200 mg/kg, 250 dpm/nmole). Four hours later the mice were sacrificed and tissue levels of covalently bound metabolites were determined as described earlier. The results, shown in Figure 2A, indicate that the lung, liver and kidney contain the largest amounts of bound metabolites; much lower levels were observed in muscle, a tissue without cytochrome P-450 MFO activity. The data in Figure 2B show that diethyl maleate

pretreatment more than tripled the levels of covalently bound metabolite(s) in lung, liver, kidney and spleen. (Significantly different from control $p < .01$ two tailed T test.) Thus the increase in naphthalene lung toxicity in animals pretreated with diethyl maleate is paralleled by an increase in the covalent binding of naphthalene metabolites to macromolecules in the lung.

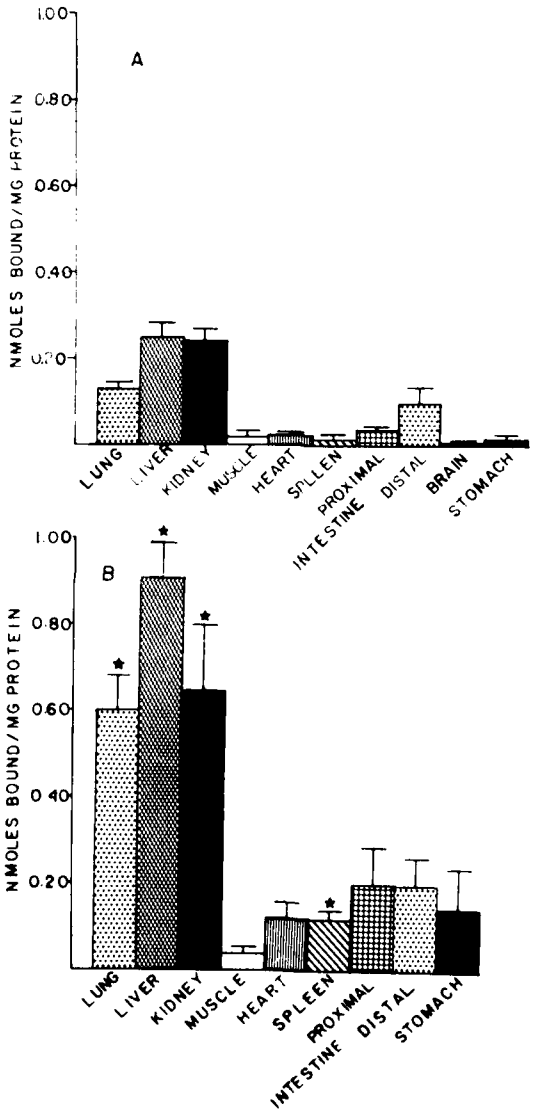


Figure 2A&B. Tissue distribution of covalent bound naphthalene metabolites in mice 4 hours after i.p. administration of 200 mg/kg 1-^{14}C-naphthalene (Panel A) or 4 hours after i.p. administration of diethyl maleate (600 µl/kg) followed 30 min later by 200 mg/kg 1-^{14}C-naphthalene. Values are the mean $\pm$ S.E. for 4 animals. * indicates a significant difference from control ($p < .01$, two tailed "T" test).

A point which must be emphasized in these studies is that the macromolecular fraction which is precipitated by trichloroacetic acid is not considered a target substance but rather is that fraction which quantitatively yields the greatest amount of bound metabolites. Binding to non-critical sites may explain why higher levels of overall covalent binding are observed in the liver even though the lung appears to be the primary site of naphthalene-induced tissue necrosis. Experiments to be conducted later will be designed to answer questions about which metabolites and which sites of binding are most critical to the toxic response.

TIME COURSE OF COVALENT BINDNG AND GLUTATHIONE DEPLETION AFTER TOXIC DOSES OF ^{14}C-NAPTHALENE

^{14}C-Naphthalene (200 mg/kg, 288 dpm/nmole) was administered intraperitoneally to groups of 4 mice each followed by sacrifice at 15, 30, 60, 120, 240 and 480 minutes. Animals serving as glutathione controls were treated with vehicle only. Lungs were perfused thoroughly with isotonic saline via the pulmonary artery and were removed and frozen along with liver, kidney and muscle. The tissues were weighed, homogenized in 3 volumes of 0.1 M phosphate buffer pH 7.4 and a 1 ml aliquot of the homogenate was transferred to a centrifuge tube containing 1.0 ml ice cold 4% sulfosalicylic acid to precipitate the tissue macromolecules. After centrifugation, an aliquot of the supernatant was taken for the determination of free sulfhydryls by the method of Ellman (1959). Glutathione standard curves run simultaneously were linear from 10 to 200 µg/ml. Covalently bound naphthalene metabolites were assayed in the precipitated macromolecular fraction.

The data in Figure 3 show that intraperitoneal administration of a toxic dose of naphthalene caused significant depletion of pulmonary and hepatic but not renal reduced glutathione. The levels of reduced glutathione in the lung decreased to a minimum of 44% of control at 4 hours but returned to nearly 80% of the control levels at the 8 hour time point. Hepatic glutathione levels decreased to a minimum at 2 hours (17% of control levels) and gradually returned to control levels at 8 hours. In contrast, renal glutathione levels are depleted only slightly by a 200 mg/kg dose of naphthalene.

The levels of covalently bound naphthalene metabolites rose to a maximum between 2 and 4 hours and inversely paralleled the decrease in glutathione content in both lung and liver. Similar to the results previously presented, the levels of covalently bound metabolites were highest in liver, with lower levels being found in lung and kidney and negligible covalent binding in the muscle.

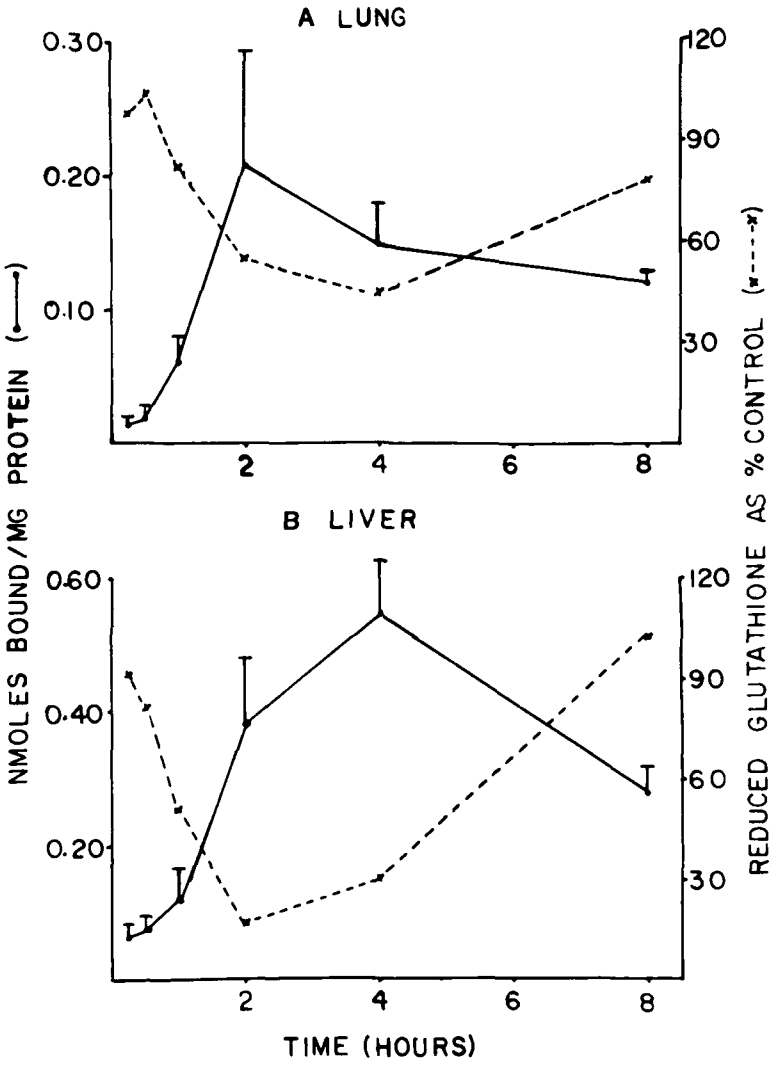
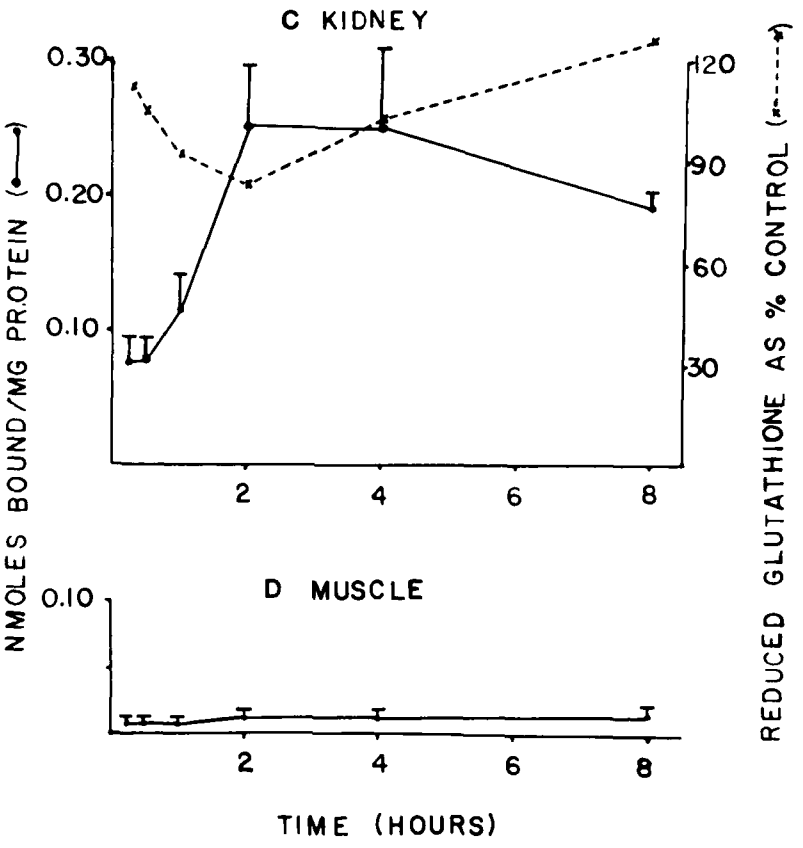


Figure 3A,B,C,D. Covalently bound naphthalene metabolites and depletion of reduced glutathione in mouse tissues at varying times after the administration of 200 mg/kg ^{14}C-naphthalene i.p. Values are the means $\pm$ S.E. for 4 animals at each time point. Values for tissue reduced glutathione are reported as a percent of that in vehicle treated controls. Standard errors were less than 12% of their respective mean.


C KIDNEY
0.30
0.20
0.10
120
90
60
30
2
4
6
8
D MUSCLE
0.10
2
4
6
8
NMOLES BOUND/MG PROTEIN (—•—)
REDUCED GLUTATHIONE AS % CONTROL (*------*)
TIME (HOURS)

These results are consistent with the idea that reactive naphthalene metabolites are being formed in mouse lung, liver and kidney and that glutathione (at least in lung and liver) is conjugating with these metabolites. These studies also indicate that the covalent binding of naphthalene metabolites precedes the tissue lesions in the lung.

ROLE OF THE LIVER IN THE FORMATION OF PULMONARY TOXIC METABOLITES

Reid et al. (1973) have suggested that the liver might play a role in the initial metabolic activation of aromatic hydrocarbons such as bromobenzene or benzo(a)pyrene (Smith and Bend, 1979) with the subsequent circulation of these metabolites to the lung (followed by either further metabolism or interaction with critical macromolecules) and that thus the liver may contribute to the toxicity and carcinogenicity of such compounds in the lung. Quantitatively the liver contains much greater amounts of cytochrome P-450 MFO activity than lung and conceivably may catalyze the formation of epoxides which circulate to the lung and overwhelm pulmonary detoxification mechanisms. This is supported by recent studies showing that the isolated perfused rat liver can metabolize benzo(a)pyrene to benzo(a)pyrene-4,5-oxide which effluxes from the liver (Smith and Bend, 1979). Moreover, studies in which ^{3}H-benzo(a)pyrene-4,5-oxide is used as a substrate in the isolated perfused lung preparation have shown that radioactivity derived from this substrate becomes covalently bound to lung tissue macromolecules during the perfusion period (Smith et al., 1980).

The possibility that the liver may play a role in the metabolism of naphthalene to a proximate reactive metabolite(s) which circulates to the lung and contributes to the lung toxicity is suggested by two factors: (1) naphthalene-1,2-epoxide, a known metabolite of naphthalene produced in liver microsomal preparations, is sufficiently stable to circulate to the lung ($T_{1/2}$ = 4-6 minutes in water) and (2) recent in vitro evidence suggests that reactive naphthalene metabolites (which bind covalently) are formed through the cytochrome P-450 catalyzed formation of 1-naphthol followed by further metabolic activation of 1-naphthol (Hesse and Metzger, 1979). Thus 1-naphthol produced by hepatic cytochrome P-450 could circulate to the lung and undergo further enzyme catalyzed metabolism to highly reactive lung toxic metabolites.

A possible approach to determine the role of the liver in the initial metabolic activation of naphthalene to metabolites which damage the lung has been suggested by recent reports showing that intraperitoneal administration of p-xylene to rats or rabbits causes a marked decrease in pulmonary but not hepatic cytochrome P-450 (Patel et al., 1978; Patel et al., 1979).

To determine whether p-xylene administration produces selective decreases in pulmonary cytochrome P-450 in the mouse as it does in the rat and rabbit, two groups of

mice were treated with either corn oil or p-xylene (1 g/kg in corn oil). Sixteen hours later the mice were sacrificed, livers and lungs were removed and placed in ice cold Tris/KCl buffer. Microsomal fractions were prepared by high speed centrifugation of 9000 xg supernatant by methods described elsewhere (Boyd et al., 1978). The final microsomal pellet was dissolved in 0.1 M phosphate buffer pH 7.4 and cytochrome P-450 content was determined by measurement of difference spectra of the carbon monoxide reduced cytochrome [method of Omura and Sato (1961)] using a Cary 210 dual beam spectrophotometer. Similar to the results obtained in the rat and rabbit, administration of p-xylene to mice causes a selective decrease in pulmonary but not hepatic cytochrome P-450 (Table 2).

TABLE 2

EFFECT OF p-XYLENE TREATMENT ON PULMONARY AND HEPATIC MICROSOMAL CYTOCHROME P-450

Tissue	Treatment	Microsomal Yield(mg/g)	Cytochrome P-450 (nmoles/mg protein)	% of Control
Lung	corn oil	4.2	0.116	
Lung	p-xylene	3.0	0.042	36.2
Liver	corn oil	13.7	1.85	
Liver	p-xylene	11.0	1.81	97.8

Experiments to determine whether doses of p-xylene cause histologically detectable lung damage are in progress. If the results of this experiment reveal no damage to the bronchiolar epithelium by p-xylene, then experiments to assess the effects of p-xylene pretreatment on naphthalene lung toxicity and metabolism to covalently bound adducts will be conducted.

SUMMARY AND CONCLUSIONS

The second section of the Comparative Biochemistry and Metabolism Subprogram has focused its efforts on Air Force compounds which cause acute lung damage. Studies on naphthalene, a volatile hydrocarbon in shale oil, have confirmed previous reports on the bronchiolar necrosis caused by intraperitoneal administration in mice. These earlier studies also have been extended to show that the pulmonary damage by naphthalene can

be prevented by pretreatment of the mice with piperonyl butoxide, a potent inhibitor of the cytochrome P-450 mixed function oxygenases. Likewise, pretreatment with diethyl maleate, which rapidly depletes tissue glutathione stores, markedly exacerbates the pulmonary damage due to a subsequently administered dose of naphthalene. These results, combined with an earlier electron microscopic study showing the Clara cell (a major locus of pulmonary cytochrome P-450) to be a primary target for the cytotoxicity of naphthalene, suggested that this hydrocarbon may undergo metabolic activation in the lung to highly electrophilic metabolites which play a role in the lung toxicity and that cellular glutathione levels are important in modulating the toxic response.

Toxic doses of ^{14}C-naphthalene result in the covalent binding of radiolabel which is highest in tissues containing cytochrome P-450 MFO activity (lung, liver and kidney) but which is low in tissues such as muscle that do not. The covalent binding is tissue dependent and precedes the tissue lesions. Covalent binding in lung, liver, kidney and spleen from animals pretreated with diethyl maleate followed by a toxic dose of ^{14}C-naphthalene is triple that found in vehicle pretreated controls.

While these results must be considered preliminary, they are consistent with the view that naphthalene is being metabolized by the cytochrome P-450 monooxygenases to highly electrophilic metabolite(s) which bind covalently to critical cellular macromolecules and play a role in the toxic response. Further studies are necessary to strengthen this view. Future experiments will be designed to further elucidate the roles of various naphthalene metabolites and macromolecular adducts in the toxic response by comparing pathways and adducts in target and non-target tissues. Such an approach may not only yield new insights about the basis for the target organ lung toxicity of naphthalene in the mouse but also in a much broader sense may yield methods for studying the abilities of human lung tissue to carry out such metabolic processes. Moreover, these basic studies may result in methods for determining whether exposure to Air Force materials like the hydrazines or jet fuels can alter the biochemical functioning of pulmonary Clara cells thereby influencing the potential toxicity of other "lifestyle" pulmonary toxins or carcinogens (such as those in cigarette smoke).

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